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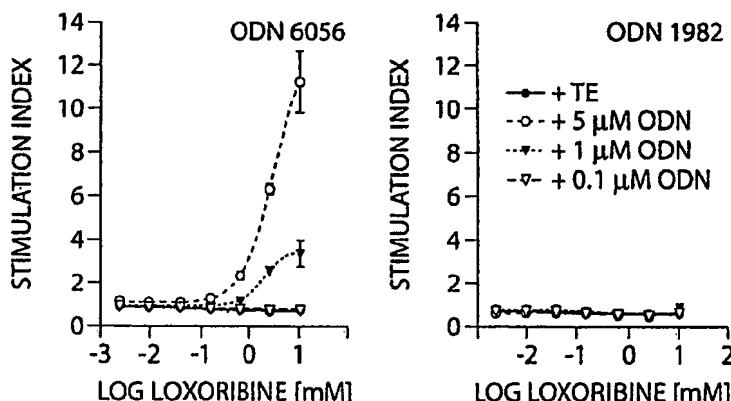
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(71) Applicants (for all designated States except US): COLEY PHARMACEUTICAL GMBH [DE/DE]; Elisabeth-Selbert-Strasse 9, 40764 Langenfeld (DE). COLEY PHARMACEUTICAL GROUP, INC. [US/US]; 93 Worcester Street, Suite 101, Wellesley, MA 02481 (US).
(72) Inventors; and
(75) Inventors/Applicants (for US only): JURK, Marion [DE/DE]; Klosterstr. 4, 41540 Dormagen (DE). VOLLMER, Joerg [DE/DE]; Flemingweg 15, 40591 Dusseldorf (DE). KRIEG, Arthur, M. [US/US]; 173 Winding River Road, Wellesley, MA 02482 (US). UHLMANN, Eugen [DE/DE]; Zum Talblick 31, 61479 Glashuetten (DE). NOLL, Bernhard, O. [DE/DE]; Dechant-Hess-Strasse 28, 41468 Neuss (DE).
(74) Agent: TREVISAN, Maria, A.; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).
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(54) Title: MODULATION OF TLR-MEDIATED IMMUNE RESPONSES USING ADAPTOR OLIGONUCLEOTIDES



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(57) Abstract: The invention relates to the ability of certain oligonucleotides to modify the profile of TLR ligands such as TLR7, TLR8 and TLR9 ligands.



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

**MODULATION OF TLR-MEDIATED IMMUNE RESPONSES USING ADAPTOR
OLIGONUCLEOTIDES**

Related Applications

This application claims priority under 35 U.S.C. §119 from U.S. provisional application serial number 60/720,981, filed September 27, 2005, entitled "MODULATION OF TLR-MEDIATED IMMUNE RESPONSES USING ADAPTOR OLIGONUCLEOTIDES", the entire contents of which are incorporated by reference herein.

Field of the Invention

The invention relates to modulation of TLR-mediated immune responses using particular nucleic acids.

Background of the Invention

Reaction to certain motifs in bacterial DNA is an important function of natural immunity. Bacterial DNA has long been known to be mitogenic for mammalian B lymphocytes (B cells), whereas mammalian DNA generally is not. The discovery that this immune recognition was directed to specific DNA sequences centered on a motif containing an unmethylated CpG dinucleotide opened the field to molecular immunologic approaches. Krieg AM et al. (1995) *Nature* 374:546-9. The immunostimulatory effects of so-called CpG DNA can be reproduced using synthetic oligodeoxynucleotides (ODN) containing CpG dinucleotides in the context of certain preferred flanking sequence, a CpG motif. CpG-containing ODN (CpG-ODN) have been reported to exert a number of effects on various types of cells of the immune system, including protecting primary B cells from apoptosis, promotion of cell cycle entry, and skewing an immune response toward a Th1-type immune response, e.g., induction of interleukin 6 (IL-6), interleukin 12 (IL-12), gamma interferon (IFN- γ), activation of antigen-specific cytolytic T lymphocytes (CTL), and induction in the mouse of IgG2a.

Recently it has been reported that the immunomodulatory effects of CpG DNA involve signaling by Toll-like receptor 9 (TLR9). It is believed that CpG DNA is internalized into a cell via a sequence-nonspecific pathway and traffics to the endosomal compartment, where it interacts with TLR9 in a sequence-specific manner. TLR9 signaling pathways lead to induction of a number of immune-function related genes, including notably NF- κ B.

The TLRs are a large family of receptors that recognize specific molecular structures that are present in pathogens (pathogen-associated molecular patterns or PAMPs) and are also termed pattern recognition receptors (PRRs). Immune cells expressing PRRs are activated upon recognition of PAMPs and trigger the generation of optimal adaptive immune responses. PRRs consisting of 10 different TLR subtypes, TLR1 to TLR10, have been described. Such TLRs have been described to be involved in the recognition of double-stranded RNA (TLR3), lipopolysaccharide (LPS) (TLR4), bacterial flagellin (TLR5), small anti-viral compounds (TLR7 and TLR8), and bacterial DNA or CpG ODN (TLR9). (See review by Uhlmann et al. (2003) *Curr Opin Drug Discov Devel* 6:204-17.) In addition, RNA molecules have been identified that are believed to interact with and signal through TLR7 and TLR8. (See International patent application PCT/US03/10406.) Such immunostimulatory RNA molecules are believed to have a base sequence that includes at least one guanine and at least one uracil. The immunostimulatory G,U-rich RNA does not require a CpG motif as described for TLR9. The corresponding class of RNA molecules found in nature is believed to be present in ribosomal RNA (rRNA), transfer RNA (tRNA), messenger RNA (mRNA), and viral RNA (vRNA).

Following the discovery of immunostimulatory CpG DNA, a number of reports appeared describing short DNA sequences with immunoinhibitory effects. It has long been known that poly-G sequences were immunoinhibitory. Published PCT patent application WO 00/14217 describes ODN containing an inhibitory motif N₁N₂GN₃G in which at least any two of N₁, N₂, and N₃ are G (guanosine). Krieg and colleagues described a group of inhibitory 15-mer ODN, having three or four consecutive G, that blocked apoptosis protection and cell-cycle entry induced by stimulatory ODN. Lenert P et al. (2001) *Antisense Nucleic Acid Drug Dev* 11:247-56; Stunz LL et al. (2002) *Eur J Immunol* 32:1212-22; Lenert P et al. (2003) *Antisense Nucleic Acid Drug Dev* 13:143-50. The immunoinhibitory effect of these ODN was reported to be specific for CpG-ODN and to involve a mechanism other than simple competition for cellular uptake. Stunz LL et al. (2002) *Eur J Immunol* 32:1212-22. Independently, Klinman and colleagues reported a single immunoinhibitory ODN. Zeuner RA et al. (2002) *Arthritis Rheum* 46:2219-24; Yamada H et al. (2002) *J Immunol* 169:5590-4.

Summary of the Invention

The invention provides methods and compositions for modulating TLR-mediated immune responses including inhibiting some responses, potentiating other responses, or some

combination thereof. The invention is premised in part on the finding that certain oligonucleotides are apparently able to change the TLR signaling profile of TLR ligands. Thus, these "adaptor" oligonucleotides are able to essentially convert TLR7 ligands into TLR8 ligands, according to one embodiment, and TLR7/8 ligands into TLR8 ligands, according to another embodiment.

Thus, in one aspect, the invention provides a method for stimulating a TLR8-mediated immune response comprising administering to a subject in need thereof a TLR7/8 ligand and an adaptor oligonucleotide in an amount effective to stimulate a TLR8-mediated immune response.

In another aspect, the invention provides a method for redirecting a TLR7-mediated immune response to a TLR8-mediated immune response comprising administering to a subject experiencing a TLR7-mediated immune response an adaptor oligonucleotide in an amount effective to redirect a TLR7-mediated immune response to a TLR8-mediated immune response.

In yet another aspect, the invention provides a composition comprising a TLR7/8 ligand and an adaptor oligonucleotide.

In one embodiment, the TLR7/8 ligand is a TLR7 ligand, such as a TLR7 specific ligand. The TLR7 ligand may be a guanosine analogue such as but not limited to a C8-substituted guanosine. The TLR7 ligand may be 3M-001. The TLR7 ligand may be an adenosine-based compound such as but not limited to 6-amino-9-benzyl-2-(3-hydroxy-propoxy)-9H-purin-8-ol, or 6-amino-9-benzyl-2-butoxy-9H-purin-8-ol. The TLR7 ligand may be 7-deaza-guanosine.

Examples of C8-substituted guanosine include 7-allyl-7,8-dihydro-8-oxo-guanosine (loxoridine), 7-thia-8-oxoguanosine (immunosine, Isatoridine, ANA245, 7-thia-8-oxo-7,8-dihydroguanosine, 5-amino-3-(β -D-ribofuranosyl)-3H,6H-thiazol[4,5-d]pyrimidine-2,7-dione), 8-mercaptoguanosine, 8-bromoguanosine, 8-methylguanosine, 8-oxo-7,8-dihydroguanosine, C8-arylaminoo-2'-deoxyguanosine, C8-propynyl-guanosine, C8- and N7-substituted guanine ribonucleosides, 7-methyl-8-oxoguanosine, 8-aminoguanosine, 8-hydroxy-2'-deoxyguanosine, 7-deaza-8-substituted guanosine, and 8-hydroxyguanosine. In some embodiments, the C8-substituted guanosine is loxoridine, immunosine, or 7-deaza guanosine.

In another embodiment, the TLR7/8 ligand is a TLR8 ligand including a TLR8 specific ligand. The TLR8 ligand may be 3M-002. In embodiments in which the ligand is a

TLR8 ligand, the TLR8-mediated immune response may be enhanced at least 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold or more over the level of immune response achieved in the absence of the adaptor oligonucleotide.

In another embodiment, the TLR7/8 ligand is a TLR7 ligand and a TLR8 ligand. An example of such a TLR 7/8 ligand is an imidazoquinoline. Examples of imidazoquinolines include imidazoquinoline amine, imidazopyridine amine, 6,7-fused cycloalkylimidazopyridine amine, 1,2 bridged imidazoquinoline amine, R-848 (S-28463 or resiquimod), 4-amino-2ethoxymethyl- α,α -dimethyl-1H-imidazo[4,5-c]quinolines-1-ethanol, 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine (R-837 or Imiquimod), or S-27609. In some important embodiments, the imidazoquinoline is R848.

The TLR7/8 ligand may be 3M-003. In embodiments in which the ligand is a TLR7 and TLR8 ligand, the TLR8-mediated immune response may be enhanced at least 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold or more over the level of TLR8-mediated immune response achieved in the absence of the adaptor oligonucleotide.

In one embodiment, the adaptor oligonucleotide comprises the formula 5' X - N₅ - X 3' or the formula 5' X - N₄ 3', wherein X can be any nucleotide and may be present or absent and N₄ and N₅ represent four or five contiguous T (thymidine), U (uracil) or A (adenine) such that every N in N₄ or N₅ is identical. Depending on the embodiment, the oligonucleotide is 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 or more nucleotides in length. In important embodiments, it comprises at least one phosphorothioate internucleotide linkage (up to and including a completely phosphorothioated backbone).

In a related embodiment, the adaptor oligonucleotide comprises the formula 5' X_a - TTTTT - X_b 3', wherein X_a and X_b can independently be any nucleotide and may be present or absent. X_a and X_b may be one or more nucleotides (e.g., 1-100 nucleotides). In one embodiment, the oligonucleotide comprises 6, 7 or more contiguous T. In one important embodiment, the adaptor oligonucleotide is a thymidine (dT) homopolymer, that is optionally 17 nucleotides in length.

In another embodiment, the adaptor oligonucleotide comprises the formula 5' X_a - UUUUU - X_b 3' wherein X_a and X_b can independently be any nucleotide and may be present or absent. X_a and X_b may be one or more nucleotides (e.g., 1-100 nucleotides). In one embodiment, the oligonucleotide may comprise 6, 7 or more contiguous U. In an important embodiment, the oligonucleotide is a uracil (dU) homopolymer, that is optionally 17 nucleotides in length.

In yet another embodiment, the adaptor oligonucleotide comprises the formula 5' X_a - AAAAAA- X_b 3' wherein X_a and X_b can independently be any nucleotide and may be present or absent. X_a and X_b may be one or more nucleotides (e.g., 1-100 nucleotides). In one embodiment, the oligonucleotide may comprise 6, 7 or more contiguous A. In an important embodiment, the oligonucleotide is an adenine (dA) homopolymer, that is optionally 17 nucleotides in length.

In still another embodiment, the adaptor oligonucleotide comprises the formula 5' C_n - T_m - C_p 3', wherein n is an integer ranging from 0-100, p is an integer ranging from 0-100, and m is an integer ranging from 0-100. In one embodiment, the sum of n and p is equal to or less than the value of m such that C content of the entire oligonucleotide is 50%, less than 50%, less than 45%, less than 40%, less than 35%, less than 30%, less than 25%, less than 20%, less than 15%, less than 10%, less than 5%, or less. In some embodiments, n ranges from 3-7, m ranges from 2-10 and p ranges from 4-8, provided the percentages cited above are satisfied. Examples include 5'C₃T₁₀C₄ 3'(SEQ ID NO: 1) and 5'C₄T₈C₅ 3' (SEQ ID NO: 2).

The adaptor oligonucleotide may comprise a CpG motif, or it may lack such a motif. The motif may be an unmethylated CpG motif.

In one embodiment, the TLR 7/8 ligand and the adaptor oligonucleotide are administered separately to a subject. In these and other embodiments, the ligand and oligonucleotide may still be administered substantially simultaneously with each other. In still another embodiment, the oligonucleotide and the TLR7/8 ligand are conjugated to each other. In one embodiment, the adaptor oligonucleotide is covalently attached to the TLR 7/8 ligand.

In one embodiment, the adaptor oligonucleotide is a DNA while in another it is an RNA. In one embodiment, the adaptor oligonucleotide is not immunostimulatory when used alone. In one embodiment, the adaptor oligonucleotide alone does not stimulate a TLR7- or TLR8-mediated immune response.

In one embodiment, the composition further comprises an antigen and/or the method further comprises administering an antigen to the subject.

In one embodiment, the subject has an infection. In another embodiment, the subject has cancer. In yet another embodiment, the subject has allergy or asthma.

The composition may be a pharmaceutical preparation further comprising a pharmaceutically acceptable carrier.

In one aspect, the invention provides a use of the TLR ligand and adaptor oligonucleotide combination, as described above, for the preparation of a medicament for vaccinating a subject.

In one aspect, the invention provides a method for preparing a vaccine. The method includes the step of placing the TLR ligand and adaptor oligonucleotide combination, as described above, in intimate association with an antigen and, optionally, a pharmaceutically acceptable carrier.

In one aspect, the invention provides a use of the TLR ligand and adaptor oligonucleotide combination, as described above, for the preparation of a medicament for treating infection in a subject.

In one aspect, the invention provides a composition useful for the treatment of infection that includes the TLR ligand and adaptor oligonucleotide combination, as described above, and an anti-microbial agent or medicament.

In one aspect, the invention provides a use of the TLR ligand and adaptor oligonucleotide combination, as described above, for the preparation of a medicament for treating cancer in a subject.

In one aspect, the invention provides a composition useful for the treatment of cancer that includes the TLR ligand and adaptor oligonucleotide combination, as described above, and a cancer medicament.

In one aspect, the invention provides a use of the TLR ligand and adaptor oligonucleotide combination, as described above, for the preparation of a medicament for treating an allergic condition in a subject.

In one aspect, the invention provides a composition useful for the treatment of an allergic condition that includes the TLR ligand and adaptor oligonucleotide combination, as described above, and an allergy medicament.

In one aspect, the invention provides a use of the TLR ligand and adaptor oligonucleotide combination, as described above, for the preparation of a medicament for treating asthma in a subject.

In one aspect, the invention provides a composition useful for the treatment of asthma that includes the TLR ligand and adaptor oligonucleotide combination, as described above, and an asthma medicament.

In other aspects, the invention provides screening methods for identifying TLR7/8 ligands and adaptor oligonucleotides. Thus, in one aspect, the invention provides a method

for identifying a TLR8 ligand comprising contacting a TLR8-expressing cell with a test ligand in the presence and absence of an adaptor oligonucleotide, and measuring stimulation of the TLR8-expressing cell in response to the test ligand in the presence and absence of the adaptor oligonucleotide. A TLR8 ligand is identified by an increased stimulation in the presence of the adaptor oligonucleotide. The increased stimulation is preferably an increase over a non-zero stimulation level in the absence of the adaptor oligonucleotide.

In another aspect, the invention provides a method for identifying a TLR7 ligand comprising contacting a TLR7-expressing cell and a TLR8-expressing cell with a test ligand in the presence and absence of an adaptor oligonucleotide, and measuring stimulation of the TLR7-expressing cell and the TLR8-expressing cell in response to the test ligand in the presence and absence of the adaptor oligonucleotide. A TLR7 ligand is identified by a decreased stimulation in the TLR7-expressing cell and an increased stimulation in the TLR8-expressing cell in the presence of the adaptor oligonucleotide. The increased stimulation, in this aspect, is preferably an increase over a zero stimulation level in the absence of the adaptor oligonucleotide.

In another aspect, the invention provides a method for identifying an adaptor oligonucleotide comprising contacting a TLR8-expressing cell with a known TLR7 ligand in the presence and absence of a test adaptor oligonucleotide, and measuring stimulation of the TLR8-expressing cell in response to the TLR7 ligand in the presence of the test adaptor oligonucleotide. The level of stimulation may be compared to the level in the absence of the test oligonucleotide, the level in the absence of the TLR7 ligand and the presence of the test oligonucleotide, and/or in the presence of the TLR7 ligand and a known adaptor oligonucleotide. An adaptor oligonucleotide is identified by increased stimulation of the TLR8-expressing cell in its presence as compared to in its absence, in one embodiment, provided that the oligonucleotide itself is not mediating a TLR8-mediated immune response (as determined by the control assays mentioned above).

The TLR7 and TLR8 ligands and the adaptor oligonucleotides can be any of those recited in the proceeding aspects and embodiments. The TLR7- or TLR8-expressing cells can be cells that naturally express TLR7 or TLR8 or they may be cells that are engineered to express (e.g., ectopically) TLR7 or TLR8. Stimulation may be measured by signaling through TLR7 or TLR8 and downstream effects thereof including but not limited to increased gene expression (e.g., as visualized using a reporter construct that is conjugated to the promoter

elements of a downstream target of TLR7 or TLR8), increased growth factor (cytokine) expression, production or secretion, and the like.

These and other aspects and embodiments of the invention will be described in greater detail herein.

Brief Description of the Drawings

FIGs. 1A-1B show the selective enhancement and inhibition of R-848-mediated activity on human TLR8 and TLR7 by ODN. FIG. 1A shows the inhibition of R-848 activity on human TLR7 by ODN 6056 (SEQ ID NO: 3) and 1982 (SEQ ID NO: 4) (for sequences see Tables 1 and 2). HEK293 cells stably expressing TLR7 and an NF- κ B luciferase reporter construct were incubated with 2 μ M R-848 in the presence or absence of indicated amounts of the oligo(dT)₁₇ homopolymer ODN 6056, or the heteropolymer ODN 1982. Activity of R-848 alone was set to 100%. All data points were assayed in triplicate and mean (+/- SD) is displayed. Results shown are one out of more than two independent experiments. FIG. 1B shows the sequence-selective enhancement of R-848 activity on human TLR8 by an oligo(dT)₁₇ ODN. HEK293 cells stably expressing hTLR8 and a NF- κ B-luciferase reporter construct were incubated with increasing amounts of R-848 in the absence or presence of 0.1, 1 and 5 μ M of the oligo(dT)₁₇ homopolymer ODN 6056, or the heteropolymer ODN 1982. Stimulation of NF- κ B activation was calculated in reference to medium background. All data points were assayed in triplicate and mean (\pm SD) is displayed. Results shown are one out of four independent experiments.

FIGs. 2A-C show the altered target specificity of the TLR7 ligands, loxoribine and 7-deaza-guanosine, when combined with an oligo(dT)₁₇ ODN. In FIG. 2A, HEK293 cells stably expressing hTLR7, hTLR8 or hTLR9 were incubated with increasing amounts of loxoribine. NF- κ B activation was measured by assaying luciferase activity 16h later and displayed as fold stimulation above medium background. In FIG. 2B, HEK293 cells expressing hTLR8 were incubated with increasing amounts of loxoribine in the absence or presence of the indicated concentrations of the homopolymer ODN 6056 or the heteropolymer ODN 1982. NF- κ B activation is given as fold induction above medium background. In FIG. 2C, increasing amounts of 7-deaza-guanosine (left panel) and inosine (right panel) were assayed on hTLR8 expressing HEK293 cells in the absence (filled circles) or presence (open circles) of 5 μ M ODN 6056 and NF- κ B stimulation above medium background was

calculated. All data points were assayed in triplicate. Shown are data from one out of three experiments.

FIGs. 3A-3B show that ODN 6056 inhibits TLR7-mediated NF- κ B activation in HEK293 cells. HEK293 cells stably expressing hTLR7 and a NF- κ B-luciferase reporter construct were incubated for 16h with 2.5 mM loxoribine (FIG. 3A) or 2 μ M R-848 (FIG. 3B) in the presence or absence of indicated concentrations of ODN 6056. Stimulation of NF- κ B-activation was calculated in reference to medium background. All data points were assayed in triplicate and mean (\pm SD) is displayed. Results shown are from one out of two independent experiments.

FIGs. 4A-4D show redirection of loxoribine-induced cytokine production by specific ODN. Human PBMC were incubated with 1 mM loxoribine in the absence or separate presence of increasing amounts of ODN 6056 and ODN 1982. Supernatants were collected 24h later and amounts of IFN- α (FIG. 4A), IL-12p40 (FIG. 4B), IFN- γ (FIG. 4C) and TNF- α (FIG. 4D) were measured by ELISA. Values represent mean of 3 donors (\pm SEM). The data are from one representative experiment out of four experiments.

FIGs. 5A-5F show stimulation of human monocytes to produce IL-12p40 and TNF- α upon coculture with loxoribine and oligo(dT)₁₇ ODN. Human PBMC were incubated with indicated amounts of loxoribine and ODN. Intracellular staining was performed using anti-IL-12p40/p70 (FIG. 5A) and anti-TNF- α (FIG. 5B) antibodies. Cells were stained simultaneously with anti-CD14 and anti-CD19 antibodies. Cells were gated for CD14-positive cells and percentage of IL-12p40/p70- or TNF- α -positive cells was calculated. Results show mean of three donors (\pm SEM). FIGs. 5C-5F display representative flow cytometry dot blots. Shown is one out of two independent experiments.

FIGs. 6A-6L show stimulation of human NK cells to produce IFN- γ upon co-culture with loxoribine and oligo(dT)₁₇ ODN 6056. Human PBMC were incubated with indicated amounts of loxoribine and ODN. Intracellular staining was performed using anti-IFN- γ antibodies. Cells were stained with anti-CD56 and anti-CD3 antibodies. Cells were gated for CD56-positive/ CD3 negative cells (NK cells). Percentage of IFN- γ -positive cells was calculated and is displayed within the flow cytometry dot blots. Shown are two representative donors out of five.

FIGs. 7A and 7B show influence of co-incubation of loxoribine with ODN on cytokine production from isolated cell populations. In FIG. 7A, monocytes were isolated from human PBMC (96% purity) using CD14 mAb and incubated with indicated amounts of

loxoridine in the presence or absence of ODN. The amount of IL-12p40 was determined in the supernatants by ELISA. Data display results for two individual donors (gray and black bars). Shown is one out of 3 similar experiments. In FIG. 7B, PDC were enriched (85% purity) from human PBMC of two donors (gray and black bars) using BDCA-4 mAb and incubated with indicated combinations of loxoridine and ODN for 24h. IFN- α was detected in the supernatants by ELISA. Shown are the data from one out of three similar experiments.

FIG. 8A shows the effect on hTLR8-LUC-293 cells incubated with 50 μ M R-848 in the presence of indicated amounts of ODN for 16h. NF- κ B stimulation was measured by assaying luciferase activity. NF- κ B activity induced by 50 μ M R-848 alone was set to 100%. FIG. 8B shows hTLR8-LUC-293 cells incubated with increasing concentrations of R-848 in the presence of 1 μ M of indicated ODN for 16h. NF- κ B stimulation was measured by assaying luciferase activity.

FIGs. 9A and 9B show TLR7 and TLR8 signaling using a combination of the TLR7 specific ligand 6-amino-9-benzyl-2-butoxy-9H-purin-8-ol and ODN 6056. FIG. 9A shows the effects of the combination on TLR7 signaling. FIG. 9B shows the effects of the combination on TLR8 signaling.

FIG. 10 shows the effect on TLR8 signaling of two different RNAs. The poly(rU)₁₈ (SEQ ID NO: 5) stimulates TLR8 in the absence of a TLR7/8 ligand. The mixed RNA oligo (SEQ ID NO: 16) does not stimulate TLR8 alone.

FIG. 11 shows the effects of two RNAs on R-848 TLR8 signaling, in the presence of a TLR7/8 ligand.

FIG. 12 shows the TLR8-mediated effects of ODN 6056 on TLR7 ligands (loxoridine, immunosine), a ligand that is both a TLR7 and a TLR8 ligand (R-848), and a compound that is not a TLR7/8 ligand (ribavirin).

FIGs. 13A-D show the structures of immunosine (A), two immunosine variants/monomers (B and C) that can be conjugated to an oligonucleotide at a 3' end or internally (B) or the 5' end (C), and 6-amino-9-benzyl-2-butoxy-9H-purin-8-ol (D).

FIG. 14 shows the structure of an immunosine-oligonucleotide conjugate comprising a linker.

¹FIGs. 15A-C show the structures of R-848 (A), CL-029 (B) and immunosine (C), and points at which linkers and/or oligonucleotides may be attached.

Detailed Description of the Invention

The invention relates in part to the discovery that certain oligonucleotides are able to modulate TLR-mediated immune responses. As used herein, modulating a TLR-mediated immune response refers to the ability to manipulate signaling of one or more TLRs. For example, it is possible according to the invention to change the TLR profile of particular TLR ligands such that in the presence of certain oligonucleotides these ligands signal through a different TLR. As another example, in the presence of certain oligonucleotides TLR ligands known to signal through more than one TLR demonstrate signaling through only one TLR, and in many instances such signaling is enhanced. In still another example, in the presence of certain oligonucleotides the efficacy and potency of TLR signaling is increased significantly. Thus, the invention makes it possible to selectively inhibit and/or enhance signaling by any one of or by any combination of TLR7, TLR8, and TLR9.

The invention is premised in part on the observation that co-incubation of the imidazoquinoline derivative Resiquimod (R-848), which by itself activates both TLR7 and TLR8, with an oligo(dT)₁₇ homopolymer oligonucleotide (ODN 6056) increased activity of R-848 on TLR8-expressing HEK 293 cells significantly, whereas TLR7-mediated signaling was abolished. Similarly, the combination of the guanosine analogue loxoribine, which by itself activates TLR7, and the oligo(dT)₁₇ ODN 6056 induced TLR8-mediated signaling in a sequence-selective fashion, and abolished TLR7-mediated signaling.

The cytokine profile induced by loxoribine in human immune cells was also altered by co-incubation with the oligo(dT)₁₇. IL-12, TNF- α or IFN- γ were highly secreted, but IFN- α production was abrogated. Although not intending to be bound by any particular mechanism, it is presumed that the observed alteration in induced cytokines is due to a shift in the cell types being activated from plasmacytoid DC by loxoribine alone, to monocytes by its combination with ODN and indicates that monocytes do not express functional TLR7.

The invention is therefore useful for modulating a range of immune responses. The invention in a specific embodiment is useful in inhibiting TLR7 signaling (and the associated TLR7-mediated immune response), optionally while inducing TLR8 signaling (and the associated TLR8-mediated immune response). In another specific embodiment, the invention provides for induction (including enhancement) of TLR8 signaling (and the associated TLR8-mediated immune response). Depending on the nature of the adaptor oligonucleotide, it is further possible to induce TLR9 signaling at the same time (and the associated TLR9-mediated immune response). For example, a combination of an adaptor oligonucleotide that

comprises a CpG motif and a TLR7 ligand such as loxoribine would induce TLR8 and TLR9 signaling (and their associated immune responses).

The invention is also useful for treating a range of conditions that would benefit from inhibition of TLR7-mediated immune responses and/or induction (including enhancement) of TLR8-mediated immune responses, optionally in the absence or presence of TLR9-mediated immune responses.

Conditions that would benefit from inhibition of TLR7-mediated immune responses include autoimmune conditions such as but not limited to lupus and rheumatoid arthritis, particularly those associated with infection by a DNA virus. Inhibition of a TLR7-mediated immune response with concomitant induction of a TLR8-mediated immune response can be used where it is desirable to enhance a T cell response that is being suppressed by T regulatory cells. Such conditions include, but are not limited to, some forms of cancer, as well as HCV, HBV and HIV infection. Combined TLR7 inhibition and TLR8 induction could also be beneficial in treating autoimmune disease or where it is desirable to induce an immune response but avoid TLR7 associated toxicity. Induction (including enhancement) of a TLR8-mediated immune response, independent of modulation of a TLR7-mediated immune response, is useful in, *inter alia*, the treatment of cancer and infections, in a vaccine or non-vaccine setting.

The invention is also useful in the treatment of conditions that would benefit from shifts in cytokine production, or where a subject is or becomes refractory to the effect of certain cytokines (e.g., IFN- α , as is sometimes observed in the treatment of viral infections).

TLRs

Toll-like receptors (TLRs) are a family of highly conserved polypeptides that play a critical role in innate immunity in mammals. Currently ten family members, designated TLR1 - TLR10, have been identified. The cytoplasmic domains of the various TLRs are characterized by a Toll-interleukin 1 (IL-1) receptor (TIR) domain. Medzhitov R et al. (1998) *Mol Cell* 2:253-8. Recognition of microbial invasion by TLRs triggers activation of a signaling cascade that is evolutionarily conserved in *Drosophila* and mammals. The TIR domain-containing adaptor protein MyD88 has been reported to associate with TLRs and to recruit IL-1 receptor-associated kinase (IRAK) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) to the TLRs. The MyD88-dependent signaling pathway is believed to lead to activation of NF- κ B transcription factors and c-Jun NH₂ terminal kinase

(Jnk) mitogen-activated protein kinases (MAPKs), critical steps in immune activation and production of inflammatory cytokines. For a review, see Aderem A et al. (2000) *Nature* 406:782-87.

TLRs are believed to be differentially expressed in various tissues and on various types of immune cells. For example, human TLR7 has been reported to be expressed in placenta, lung, spleen, lymph nodes, tonsil and on plasmacytoid precursor dendritic cells (pDCs). Chuang T-H et al. (2000) *Eur Cytokine Netw* 11:372-8; Kadewaki N et al. (2001) *J Exp Med* 194:863-9. Human TLR8 has been reported to be expressed in lung, peripheral blood leukocytes (PBL), placenta, spleen, lymph nodes, and on monocytes. Kadewaki N et al. (2001) *J Exp Med* 194:863-9; Chuang T-H et al. (2000) *Eur Cytokine Netw* 11:372-8. Human TLR9 is reportedly expressed in spleen, lymph nodes, bone marrow, PBL, and on pDCs and B cells. Kadewaki N et al. (2001) *J Exp Med* 194:863-9; Bauer S et al. (2001) *Proc Natl Acad Sci USA* 98:9237-42; Chuang T-H et al. (2000) *Eur Cytokine Netw* 11:372-8.

Nucleotide and amino acid sequences of human and murine TLR7 are known. See, for example, GenBank Accession Nos. AF240467, AF245702, NM_016562, AF334942, NM_133211; and AAF60188, AAF78035, NP_057646, AAL73191, and AAL73192, the contents of all of which are incorporated herein by reference. Human TLR7 is reported to be 1049 amino acids long. Murine TLR7 is reported to be 1050 amino acids long. TLR7 polypeptides include an extracellular domain having a leucine-rich repeat region, a transmembrane domain, and an intracellular domain that includes a TIR domain.

Nucleotide and amino acid sequences of human and murine TLR8 are known. See, for example, GenBank Accession Nos. AF246971, AF245703, NM_016610, XM_045706, AY035890, NM_133212; and AAF64061, AAF78036, NP_057694, XP_045706, AAK62677, and NP_573475, the contents of all of which are incorporated herein by reference. Human TLR8 is reported to exist in at least two isoforms, one that is 1041 amino acids long and another that is 1059 amino acids long. Murine TLR8 is 1032 amino acids long. TLR8 polypeptides include an extracellular domain having a leucine-rich repeat region, a transmembrane domain, and an intracellular domain that includes a TIR domain.

Nucleotide and amino acid sequences of human and murine TLR9 are known. See, for example, GenBank Accession Nos. NM_017442, AF259262, AB045180, AF245704, AB045181, AF348140, AF314224, NM_031178; and NP_059138, AAF72189, BAB19259, AAF78037, BAB19260, AAK29625, AAK28488, and NP_112455, the contents of all of which are incorporated herein by reference. Human TLR9 is reported to exist in at least two

isoforms, one that is 1032 amino acids long and another that is 1055 amino acids. Murine TLR9 is 1032 amino acids long. TLR9 polypeptides include an extracellular domain having a leucine-rich repeat region, a transmembrane domain, and an intracellular domain that includes a TIR domain.

TLR-mediated immune responses

As used herein, the term "TLR signaling" refers to any aspect of intracellular signaling associated with signaling through a TLR. As used herein, the term "TLR-mediated immune response" refers to the immune response that is associated with (e.g., is the result of) TLR signaling.

A TLR7-mediated immune response is a response associated with TLR7 signaling. TLR7-mediated immune response is generally characterized by the induction of IFN- α and IFN-inducible cytokines such as IP-10 and I-TAC. The levels of cytokines IL-1 α/β , IL-6, IL-8, MIP-1 α/β and MIP-3 α/β induced in a TLR7-mediated immune response are less than those induced in a TLR8-mediated immune response.

A TLR8-mediated immune response is a response associated with TLR8 signaling. This response is characterized by the induction of pro-inflammatory cytokines such as IFN- γ , IL-12p40/70, TNF- α , IL-1 α/β , IL-6, IL-8, MIP-1 α/β and MIP-3 α/β .

A TLR9-mediated immune response is a response associated with TLR9 signaling. This response is characterized at least by the production and/or secretion of IFN- γ and IL-12, albeit at levels lower than are achieved via a TLR8-mediated immune response.

TLR 7/8 ligand

As used herein, a "TLR7/8 ligand" collectively refers to any agent that is capable of increasing TLR7 and/or TLR8 signaling (i.e., an agonist of TLR7 and/or TLR8). Thus, when used in the absence of the adaptor oligonucleotides of the invention, some TLR7/8 ligands induce TLR7 signaling alone (e.g., TLR7 specific ligands), some induce TLR8 signaling alone (e.g., TLR8 specific ligands), and others induce both TLR7 and TLR8 signaling. It has been found according to the invention that when such ligands are combined with adaptor oligonucleotides, the TLR signaling profile of these ligands is altered. In some instances, TLR7 signaling is inhibited and TLR8 signaling is induced. In other instances, TLR8 signaling is induced (i.e., increased from a background level) or enhanced (i.e., increased

from a non-background level), and in still other embodiments, both TLR8 and TLR9 signaling are induced or enhanced.

As used herein, the term “TLR7 ligand” refers to any agent that is capable of increasing TLR7 signaling (i.e., an agonist of TLR7). In this respect, the level of TLR7 signaling may be enhanced over a pre-existing level of signaling or it may be induced over a background level of signaling. TLR7 ligands include, without limitation, guanosine analogues such as C8-substituted guanosines, mixtures of ribonucleosides consisting essentially of G and U, guanosine ribonucleotides and RNA or RNA-like molecules (PCT/US03/10406), and adenosine-based compounds (e.g., 6-amino-9-benzyl-2-(3-hydroxy-propoxy)-9H-purin-8-ol (CL-029, Sumitomo), 6-amino-9-benzyl-2-butoxy-9H-purin-8-ol (shown in FIG. 13D), and other related compounds such as those described in US 6310070 B1). TLR7 ligands are also disclosed in Gorden et al. J. Immunol. 2005, 174:1259-1268 (e.g., 3M-001, *N*-[4-(4-amino-2-ethyl-1H-imidazo[4,5-c]quinolin-1-yl)butyl-]methanesulfonamide; C₁₇H₂₃N₅O₂S; mw 361).

As used herein, the term “guanosine analogues” refers to a guanosine-like nucleotides (excluding guanosine) having a chemical modification involving the guanine base, guanosine nucleoside sugar, or both the guanine base and the guanosine nucleoside sugar. Guanosine analogues specifically include, without limitation, 7-deaza-guanosine.

Guanosine analogues further include C8-substituted guanosines such as 7-thia-8-oxoguanosine (immunosine), 8-mercaptoguanosine, 8-bromoguanosine, 8-methylguanosine, 8-oxo-7,8-dihydroguanosine, C8-arylamino-2'-deoxyguanosine, C8-propynyl-guanosine, C8- and N7- substituted guanine ribonucleosides such as 7-allyl-8-oxoguanosine (loxoribine) and 7-methyl-8-oxoguanosine, 8-aminoguanosine, 8-hydroxy-2'-deoxyguanosine, 8-hydroxyguanosine, and 7-deaza 8-substituted guanosine.

As used herein, the term “TLR8 ligand” refers to any agent that is capable of increasing TLR8 signaling (i.e., an agonist of TLR8). In this respect, the level of TLR8 signaling may be enhanced over a pre-existing level of signaling or it may be induced over a background level of signaling. TLR8 ligands include mixtures of ribonucleosides consisting essentially of G and U, guanosine ribonucleotides and RNA or RNA-like molecules (PCT/US03/10406). Additional TLR8 ligands are also disclosed in Gorden et al. J. Immunol. 2005, 174:1259-1268 (e.g., 3M-002, 2-propylthiazolo[4,5-c]quinolin-4-amine; C₁₃H₁₃N₃S; mw 243).

Some TLR7/8 ligands are ligands of both TLR7 and TLR8. These include imidazoquinolines, mixtures of ribonucleosides consisting essentially of G and U, guanosine ribonucleotides, and RNA or RNA-like molecules (PCT/US03/10406). Additional TLR7/8 ligands are also disclosed in Gorden et al. J. Immunol. 2005, 174:1259-1268 (e.g., 3M-003, 4-amino-2-(ethoxymethyl)- α,α -dimethyl-6,7,8,9-tetrahydro-1H-imidazo[4,5-c]quinoline-1-ethanol hydrate; C₁₇H₂₆N₄O₂; mw 318).

Imidazoquinolines are immune response modifiers thought to induce expression of several cytokines including interferons (e.g., IFN- α), TNF- α and some interleukins (e.g., IL-1, IL-6 and IL-12). Imidazoquinolines are capable of stimulating a Th1 immune response, as evidenced in part by their ability to induce increases in IgG2a levels. Imidazoquinoline agents reportedly are also capable of inhibiting production of Th2 cytokines such as IL-4, IL-5, and IL-13. Some of the cytokines induced by imidazoquinolines are produced by macrophages and dendritic cells. Some species of imidazoquinolines have been reported to increase NK cell lytic activity and to stimulate B cell proliferation and differentiation, thereby inducing antibody production and secretion.

As used herein, imidazoquinolines include imidazoquinoline amines, imidazopyridine amines, 6,7-fused cycloalkylimidazopyridine amines, and 1,2 bridged imidazoquinoline amines. These compounds have been described in U.S. Patent Nos. 4689338, 4929624, 5238944, 5266575, 5268376, 5346905, 5352784, 5389640, 5395937, 5494916, 5482936, 5525612, 6039969 and 6110929. Particular species of imidazoquinolines include R-848 (S-28463); 4-amino-2ethoxymethyl- α,α -dimethyl-1H-imidazo[4,5-c]quinolines-1-ethanol; 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine (R-837 or Imiquimod), and S-27609. Imiquimod is currently used in the topical treatment of warts such as genital and anal warts and has also been tested in the topical treatment of basal cell carcinoma.

As used herein, the term "TLR9 ligand" refers to any agent that is capable of increasing TLR9 signaling (i.e., an agonist of TLR9). TLR9 ligands specifically include, without limitation, immunostimulatory nucleic acids, and in particular CpG immunostimulatory nucleic acids.

As used herein, the term "immunostimulatory CpG nucleic acids" refers to any CpG-containing nucleic acid that is capable of activating an immune cell. At least the C of the CpG dinucleotide is typically, but not necessarily, unmethylated. Immunostimulatory CpG nucleic acids are described in a number of issued patents and published patent applications,

including U.S. Pat. Nos. 6,194,388; 6,207,646; 6,218,371; 6,239,116; 6,339,068; 6,406,705; and 6,429,199.

In some embodiments, the TLR ligand is a specific ligand. As used herein, a TLR7 specific ligand is one that, when used in the absence of the adaptor oligonucleotides of the invention, signals through TLR7 but not TLR8 or TLR9. Similarly, a TLR8 specific ligand is one that, when used in the absence of the adaptor oligonucleotides of the invention, signals through TLR8 but not TLR7 or TLR9. Preferably, a TLR7 specific ligand signals through TLR7 and no other TLRs, when used in the absence of the adaptor oligonucleotide, and a TLR8 specific ligand signals through TLR8 and no other TLRs, when used in the absence of the adaptor oligonucleotide.

In some embodiments, the TLR ligand is not an RNA.

The use of TLR7/8 ligands with adaptor oligonucleotides may stimulate both a TLR8 and a TLR9, resulting in downstream effects from both receptors concurrently. For example, stimulation of the TLR9 by the oligonucleotide may result in for example IFN-alpha production while stimulation of TLR8 by the oligonucleotide may result in for example IL-12, TNF-alpha and IFN-gamma production. The ligands and oligonucleotides may be conjugated to each other or may be physically separate from each other.

Adaptor oligonucleotides

According to the invention, TLR7/8 ligands are used in combination with adaptor oligonucleotides. An adaptor oligonucleotide, as used herein, is an oligonucleotide which when used with a TLR7/8 ligand modulates the activity of that ligand by inhibiting TLR7 signaling by a TLR7 ligand, inducing TLR8 signaling by a TLR7 ligand, and/or enhancing TLR8 signaling by a ligand that is both a TLR7 and a TLR8 ligand.

As used herein, the term oligonucleotide is used interchangeably with the term nucleic acid. Preferably, the term excludes plasmids, vectors and/or anti-sense nucleic acids. In some embodiments, the oligonucleotide may be immunostimulatory while in other embodiments it may be non-immunostimulatory when used alone. The oligonucleotides may be any length but preferably are 7 or 8 up to 100 nucleotides in length.

One broad class of adaptor oligonucleotides comprises the formula 5' X - N₄ - X 3' or 5' X - N₅ - X 3' wherein X can be any nucleotide and may be present or absent and N₄ and N₅ represents four and five contiguous T (thymidine), U (uracil), or A (adenine) such that every N in N₄ or N₅ is identical. The oligonucleotide may be 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17

or more nucleotides in length. It preferably comprises at least one phosphorothioate internucleotide linkage (up to and including a completely phosphorothioated backbone).

Thus, one class of adaptor oligonucleotides comprises the formula 5'X_a - TTTTT - X_b 3', wherein X_a and X_b can independently be any nucleotide and may be present or absent. X_a and X_b may represent one or more nucleotides (e.g., 1-100 nucleotides). The oligonucleotide may be 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or more nucleotides in length. The oligonucleotide may comprise 6, 7 or more contiguous T. Preferably, the adaptor oligonucleotide is a dT homopolymer (i.e., oligo dT of a length recited herein). Even more preferably, the adaptor oligonucleotide is a thymidine (dT) homopolymer 17 nucleotides in length. Most preferably, it comprises at least one phosphorothioated internucleotide linkage (up to and including a completely phosphorothioated backbone).

The adaptor oligonucleotide may be comprised of 100% T, 99% T, 98% T, 97% T, 96% T, 95% T, 94% T, 93% T, 92% T, 91% T, 90% T, 85% T, 80% T, 75% T, 70% T, 65% T, 60% T, 55% T, 50% T, 45% T or less, depending on the embodiment.

Another class of adaptor oligonucleotides comprises the formula 5' X_a - UUUUU - X_b 3' wherein X_a and X_b can independently be any nucleotide and may be present or absent. X_a and X_b may represent one or more nucleotides (e.g., 1-100 nucleotides). The oligonucleotide may be 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or more nucleotides in length. The oligonucleotide may comprise 6, 7 or more contiguous U. In an important embodiment, the oligonucleotide is a dU homopolymer that is preferably 17 nucleotides in length and having at least one phosphorothioated internucleotide linkage (up to and including a completely phosphorothioated backbone).

The adaptor oligonucleotide may be comprised of 100% U, 99% U, 98% U, 97% U, 96% U, 95% U, 94% U, 93% U, 92% U, 91% U, 90% U, 85% U, 80% U, 75% U, 70% U, 65% U, 60% U, 55% U, 50% U, 45% U or less, depending on the embodiment.

Yet, another class of adaptor oligonucleotides comprises the formula 5' X_a - AAAAA - X_b 3' wherein X_a and X_b can independently be any nucleotide and may be present or absent. X_a and X_b may represent one or more nucleotides (e.g., 1-100 nucleotides). The oligonucleotide may be 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or more nucleotides in length. The oligonucleotide may comprise 6, 7 or more contiguous A. In an important embodiment, the oligonucleotide is a dA homopolymer that is preferably 17 nucleotides in length and having at least one phosphorothioated internucleotide linkage (up to and including a completely phosphorothioated backbone).

The adaptor oligonucleotide may be comprised of 100% A, 99% A, 98% A, 97% A, 96% A, 95% A, 94% A, 93% A, 92% A, 91% A, 90% A, 85% A, 80% A, 75% A, 70% A, 65% A, 60% A, 55% A, 50% A, 45% A or less, depending on the embodiment.

Another class of adaptor oligonucleotides comprises the formula 5' C_n - T_m - C_p 3', wherein n is an integer ranging from 0-100 (e.g., 3-7), p is an integer ranging from 0-100 (e.g., 4-8), and m is an integer ranging from 0-100 (e.g., 2-10). Preferably, the sum of n and p is equal to or less than the value of m such that C content is 50%, less than 50%, less than 45%, less than 40%, less than 35%, less than 30%, less than 25%, less than 20%, less than 15%, less than 10%, less than 5%, or less. In some embodiments, n ranges from 3-7, m ranges from 2-10 and p ranges from 4-8, provided the percentages cited above are satisfied. Some species of this formula are shown in FIG. 8. Examples include 5' C₃T₁₀C₄ 3' (SEQ ID NO: 1), 5' C₄T₈C₅ 3' (SEQ ID NO: 2), 5' C₅T₆C₆ 3' (SEQ ID NO: 6), 5' C₆T₄C₇ 3' (SEQ ID NO: 7), and 5' C₇T₂C₈ 3' (SEQ ID NO: 8).

In some embodiments, the adaptor oligonucleotides comprise an immunostimulatory CpG motif. The motif may be methylated or unmethylated. In the latter instance, the entire oligonucleotide can be unmethylated or portions may be unmethylated but at least the C of the 5' CG 3' must be unmethylated. The unmethylated motif and its effects on immune modulation have been described extensively in U.S. Patents such as US 6,194,388 B1; US 6,207,646 B1; US 6,239,116 B1; and US 6,218,371 B1; and published patent applications, such as PCT/US98/03678, PCT/US98/10408, PCT/US98/04703, and PCT/US99/09863. The entire contents of each of these patents and patent applications is hereby incorporated by reference.

As stated above, the terms "oligonucleotide" and "nucleic acid" are used interchangeably to mean multiple nucleotides (i.e., molecules comprising a sugar (e.g., ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a pyrimidine (e.g., cytosine (C), thymidine (T) or uracil (U)) or a purine (e.g., adenine (A) or guanine (G)). Thus, the term embraces both DNA and RNA oligonucleotides. The terms shall also include polynucleosides (i.e., a polynucleotide minus the phosphate) and any other organic base containing polymer. Oligonucleotides can be obtained from existing nucleic acid sources (e.g., genomic or cDNA), but are preferably synthetic (e.g., produced by a nucleic acid synthesizer).

The oligonucleotides can be double-stranded or single-stranded. In certain embodiments, when the oligonucleotide is single stranded, it is capable of forming secondary

and tertiary structures (e.g., by folding back on itself, or by hybridizing with itself either throughout its entirety or at select segments along its length). Accordingly, while the primary structure of such an oligonucleotide may be single stranded, its higher order structures may be double or triple stranded.

The oligonucleotides may range in length but they are preferably equal to or less than 100 nucleotides (or bases) in length. The adaptor oligonucleotides are preferably not plasmids or vectors. They are also preferably not capable of anti-sense activity, or at least the effects manifest according to the invention are not related to any anti-sense activity.

The oligonucleotides may have modified backbones. For example, they may comprise at least one internucleotide linkage which is not a phosphodiester linkage. Such a linkage may be a phosphorothioate linkage. In some embodiments, the oligonucleotides may have chimeric backbones (i.e., backbones comprised of at least two different types of internucleotide linkages).

As used herein, the term "phosphorothioate backbone" refers to a stabilized sugar phosphate backbone of an oligonucleotide in which a non-bridging phosphate oxygen is replaced by sulfur at at least one internucleotide linkage. In one embodiment a non-bridging phosphate oxygen is replaced by sulfur at each and every internucleotide linkage.

Source and Preparation of Oligonucleotides

The oligonucleotides of the instant invention can encompass various chemical modifications and substitutions, in comparison to natural RNA and DNA, involving a phosphodiester internucleoside bridge, a β -D-ribose unit and/or a natural nucleoside base (adenine, guanine, cytosine, thymine, uracil). Examples of chemical modifications are known to the skilled person and are described, for example, in Uhlmann E et al. (1990) *Chem Rev* 90:543; "Protocols for Oligonucleotides and Analogs" Synthesis and Properties & Synthesis and Analytical Techniques, S. Agrawal, Ed, Humana Press, Totowa, USA 1993; Crooke ST et al. (1996) *Annu Rev Pharmacol Toxicol* 36:107-29; and Hunziker J et al. (1995) *Mod Synth Methods* 7:331-417. An oligonucleotide according to the invention may have one or more modifications, wherein each modification is located at a particular phosphodiester internucleoside bridge and/or at a particular β -D-ribose unit and/or at a particular natural nucleoside base position in comparison to an oligonucleotide of the same sequence which is composed of natural DNA or RNA.

For example, the oligonucleotides may include one or more modifications and wherein each modification is independently selected from:

- a) the replacement of a phosphodiester internucleoside bridge located at the 3' and/or the 5' end of a nucleoside by a modified internucleoside bridge,
- b) the replacement of phosphodiester bridge located at the 3' and/or the 5' end of a nucleoside by a dephospho bridge,
- c) the replacement of a sugar phosphate unit from the sugar phosphate backbone by another unit,
- d) the replacement of a β -D-ribose unit by a modified sugar unit, and
- e) the replacement of a natural nucleoside base by a modified nucleoside base.

More detailed examples for the chemical modification of an oligonucleotide follow.

The oligonucleotides may include modified internucleotide linkages, such as those described in (a) or (b) above. These modified linkages may be partially resistant to degradation (e.g., are stabilized). A "stabilized oligonucleotide" shall mean an oligonucleotide that is relatively resistant to in vivo degradation (e.g., via an exo- or endo-nuclease) resulting from such modifications. Oligonucleotides having phosphorothioate linkages, in some embodiments, may provide maximal activity and protect the oligonucleotide from degradation by intracellular exo- and endo-nucleases.

A phosphodiester internucleoside bridge located at the 3' and/or the 5' end of a nucleoside can be replaced by a modified internucleoside bridge, wherein the modified internucleoside bridge is for example selected from phosphorothioate, phosphorodithioate, NR¹R²-phosphoramidate, boranophosphate, α -hydroxybenzyl phosphonate, phosphate-(C₁-C₂₁)-O-alkyl ester, phosphate-[(C₆-C₁₂)aryl-(C₁-C₂₁)-O-alkyl]ester, (C₁-C₈)alkylphosphonate and/or (C₆-C₁₂)arylphosphonate bridges, (C₇-C₁₂)- α -hydroxymethyl-aryl (e.g., disclosed in WO 95/01363), wherein (C₆-C₁₂)aryl, (C₆-C₂₀)aryl and (C₆-C₁₄)aryl are optionally substituted by halogen, alkyl, alkoxy, nitro, cyano, and where R¹ and R² are, independently of each other, hydrogen, (C₁-C₁₈)-alkyl, (C₆-C₂₀)-aryl, (C₆-C₁₄)-aryl-(C₁-C₈)-alkyl, preferably hydrogen, (C₁-C₈)-alkyl, preferably (C₁-C₄)-alkyl and/or methoxyethyl, or R¹ and R² form, together with the nitrogen atom carrying them, a 5-6-membered heterocyclic ring which can additionally contain a further heteroatom from the group O, S and N.

The phosphodiester bridge located at the 3' and/or the 5' end of a nucleoside can be replaced by a dephospho bridge (dephospho bridges are described, for example, in Uhlmann E

and Peyman A in "Methods in Molecular Biology", Vol. 20, "Protocols for Oligonucleotides and Analogs", S. Agrawal, Ed., Humana Press, Totowa 1993, Chapter 16, pp. 355 ff), wherein a dephospho bridge is for example selected from the dephospho bridges formacetal, 3'-thioformacetal, methylhydroxylamine, oxime, methylenedimethyl-hydrazo, dimethylenesulfone and/or silyl groups.

A sugar phosphate unit (i.e., a β -D-ribose and phosphodiester internucleoside bridge together forming a sugar phosphate unit) from the sugar phosphate backbone (i.e., a sugar phosphate backbone is composed of sugar phosphate units) can be replaced by another unit, wherein the other unit is for example suitable to build up a "morpholino-derivative" oligomer (as described, for example, in Stinchak EP et al. (1989) *Nucleic Acids Res* 17:6129-41), that is, for example, the replacement with a morpholino-derivative unit, or to build up a polyamide nucleic acid ("PNA"; as described for example, in Nielsen PE et al. (1994) *Bioconjug Chem* 5:3-7), that is, for example, the replacement with a PNA backbone unit, e.g., by 2-aminoethylglycine. The oligonucleotide may have other carbohydrate backbone modifications and replacements, such as peptide nucleic acids with phosphate groups (PHONA), locked nucleic acids (LNA), and oligonucleotides having backbone sections with alkyl linkers or amino linkers. The alkyl linker may be branched or unbranched, substituted or unsubstituted, and chirally pure or a racemic mixture.

The β -ribose unit or a β -D-2'-deoxyribose unit can be replaced by a modified sugar unit, wherein the modified sugar unit is for example selected from β -D-ribose, α -D-2'-deoxyribose, L-2'-deoxyribose, 2'-F-2'-deoxyribose, 2'-F-arabinose, 2'-O-(C₁-C₆)alkyl-ribose, 2'-O-methylribose, 2'-O-(C₂-C₆)alkenyl-ribose, 2'-[O-(C₁-C₆)alkyl-O-(C₁-C₆)alkyl]-ribose, 2'-NH₂-2'-deoxyribose, β -D-xylo-furanose, α -arabinofuranose, 2,4-dideoxy- β -D-erythro-hexopyranose, and carbocyclic (described, for example, in Froehler (1992) *J Am Chem Soc* 114:8320) and/or open-chain sugar analogs (described, for example, in Vandendriessche et al. (1993) *Tetrahedron* 49:7223) and/or bicyclosugar analogs (described, for example, in Tarkov M et al. (1993) *Helv Chim Acta* 76:481).

In some embodiments, the modified sugar is a 2' modified ribose. In some embodiments the sugar is 2'-O-methylribose, particularly for one or both nucleotides linked by a phosphodiester or phosphodiester-like internucleoside linkage.

Nucleic acids also include substituted purines and pyrimidines such as C-5 propyne pyrimidine and 7-deaza-7-substituted purine modified bases. Wagner RW et al. (1996) *Nat Biotechnol* 14:840-4. Purines and pyrimidines include but are not limited to adenine,

cytosine, guanine, and thymine, and other naturally and non-naturally occurring nucleobases, substituted and unsubstituted aromatic moieties.

A modified base is any base which is chemically distinct from the naturally occurring bases typically found in DNA and RNA such as T, C, G, A, and U, but which share basic chemical structures with these naturally occurring bases. The modified nucleoside base may be, for example, selected from hypoxanthine, uracil, dihydrouracil, pseudouracil, 2-thiouracil, 4-thiouracil, 5-aminouracil, 5-(C₁-C₆)-alkyluracil, 5-(C₂-C₆)-alkenyluracil, 5-(C₂-C₆)-alkynyluracil, 5-(hydroxymethyl)uracil, 5-chlorouracil, 5-fluorouracil, 5-bromouracil, 5-hydroxycytosine, 5-(C₁-C₆)-alkylcytosine, 5-(C₂-C₆)-alkenylcytosine, 5-(C₂-C₆)-alkynylcytosine, 5-chlorocytosine, 5-fluorocytosine, 5-bromocytosine, N²-dimethylguanine, 2,4-diamino-purine, 8-azapurine, a substituted 7-deazapurine, preferably 7-deaza-7-substituted and/or 7-deaza-8-substituted purine, 5-hydroxymethylcytosine, N4-alkylcytosine, e.g., N4-ethylcytosine, 5-hydroxydeoxycytidine, 5-hydroxymethyldeoxycytidine, N4-alkyldeoxycytidine, e.g., N4-ethyldeoxycytidine, 6-thiodeoxyguanosine, and deoxyribonucleosides of nitropyrrrole, C5-propynylpyrimidine, and diaminopurine e.g., 2,6-diaminopurine, inosine, 5-methylcytosine, 2-aminopurine, 2-amino-6-chloropurine, hypoxanthine or other modifications of a natural nucleoside bases. This list is meant to be exemplary and is not to be interpreted to be limiting.

In particular formulae described herein modified bases may be incorporated. For example, a cytosine may be replaced with a modified cytosine. A modified cytosine as used herein is a naturally occurring or non-naturally occurring pyrimidine base analog of cytosine which can replace this base without impairing the immunostimulatory activity of the oligonucleotide. Modified cytosines include but are not limited to 5-substituted cytosines (e.g., 5-methyl-cytosine, 5-fluoro-cytosine, 5-chloro-cytosine, 5-bromo-cytosine, 5-iodo-cytosine, 5-hydroxy-cytosine, 5-hydroxymethyl-cytosine, 5-difluoromethyl-cytosine, and unsubstituted or substituted 5-alkynyl-cytosine), 6-substituted cytosines, N4-substituted cytosines (e.g., N4-ethyl-cytosine), 5-aza-cytosine, 2-mercpto-cytosine, isocytosine, pseudo-isocytosine, cytosine analogs with condensed ring systems (e.g., N,N'-propylene cytosine or phenoxazine), and uracil and its derivatives (e.g., 5-fluoro-uracil, 5-bromo-uracil, 5-bromovinyl-uracil, 4-thio-uracil, 5-hydroxy-uracil, 5-propynyl-uracil). Some of the preferred cytosines include 5-methyl-cytosine, 5-fluoro-cytosine, 5-hydroxy-cytosine, 5-hydroxymethyl-cytosine, and N4-ethyl-cytosine. In another embodiment of the invention, the

cytosine base is substituted by a universal base (e.g., 3-nitropyrrole, P-base), an aromatic ring system (e.g., fluorobenzene or difluorobenzene) or a hydrogen atom (dSpacer).

As another example, a guanine may be replaced with a modified guanine base. A modified guanine as used herein is a naturally occurring or non-naturally occurring purine base analog of guanine which can replace this base without impairing the immunostimulatory activity of the oligonucleotide. Modified guanines include but are not limited to 7-deazaguanine, 7-deaza-7-substituted guanine (such as 7-deaza-7-(C2-C6)alkynylguanine), 7-deaza-8-substituted guanine, hypoxanthine, N2-substituted guanines (e.g., N2-methyl-guanine), 5-amino-3-methyl-3H,6H-thiazolo[4,5-d]pyrimidine-2,7-dione, 2,6-diaminopurine, 2-aminopurine, purine, indole, adenine, substituted adenines (e.g., N6-methyl-adenine, 8-oxo-adenine), 8-substituted guanine (e.g., 8-hydroxyguanine and 8-bromoguanine), and 6-thioguanine. In another embodiment of the invention, the guanine base is substituted by a universal base (e.g., 4-methyl-indole, 5-nitro-indole, and K-base), an aromatic ring system (e.g., benzimidazole or dichloro- benzimidazole, 1-methyl-1H-[1,2,4]triazole-3-carboxylic acid amide) or a hydrogen atom (dSpacer).

For use in the instant invention, the oligonucleotides of the invention can be synthesized de novo using any of a number of procedures well known in the art including, for example, the β -cyanoethyl phosphoramidite method (Beaucage SL et al. (1981) *Tetrahedron Lett* 22:1859), or the nucleoside H-phosphonate method (Garegg et al. (1986) *Tetrahedron Lett* 27:4051-4; Froehler BC et al. (1986) *Nucleic Acids Res* 14:5399-407; Garegg et al. (1986) *Tetrahedron Lett* 27:4055-8; Gaffney et al. (1988) *Tetrahedron Lett* 29:2619-22). These chemistries can be performed by a variety of commercially available automated nucleic acid synthesizers. These oligonucleotides are referred to as synthetic oligonucleotides. An isolated oligonucleotide generally refers to an oligonucleotide which is separated from components which it is normally associated with in nature. As an example, an isolated oligonucleotide may be one which is separated from a cell, from a nucleus, from mitochondria or from chromatin.

Modified backbones such as phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl-and alkyl-phosphonates can be made, for example, as described in U.S. Pat. No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Pat. No. 5,023,243 and European Patent No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA

backbone modifications and substitutions have been described (e.g., Uhlmann E et al. (1990) *Chem Rev* 90:544; Goodchild J (1990) *Bioconjugate Chem* 1:165).

Isolated

In certain embodiments the TLR7/8 ligands and/or the adaptor oligonucleotides are isolated. An isolated ligand or oligonucleotide is a ligand or oligonucleotide that is substantially pure or is free of other substances with which it is ordinarily found in nature or in *in vivo* systems to an extent practical and appropriate for its intended use. In particular, the ligand or oligonucleotide is sufficiently pure and is sufficiently free from other biological constituents of cells so as to be useful in, for example, producing pharmaceutical preparations. Because an isolated ligand or oligonucleotide may be admixed with a pharmaceutically acceptable carrier in a pharmaceutical preparation, the ligand or oligonucleotide may comprise only a small percentage by weight of the preparation. The ligand or oligonucleotide is nonetheless isolated in that it has been substantially separated from the substances with which it may be associated in living systems.

Conjugates

As used herein, the term "conjugate" refers to any combination of two or more component parts that are linked together, directly or indirectly, via any physicochemical interaction. In one embodiment the conjugate is a combination of two or more component parts that are linked together, directly or indirectly, via covalent bonding.

In one aspect the invention provides a composition including a conjugate of a TLR ligand and an adaptor oligonucleotide. In one embodiment the conjugate is made via a covalent bond. In one embodiment the conjugate comprises a linker.

The conjugate can include one or more adaptor oligonucleotides and one or more TLR7/8 ligands of the invention. The conjugate can, alternatively or additionally, include one or more other molecules including but not limited to antigens or other medicaments.

FIGs. 13-15 illustrate many features of such conjugates including points of attachment on both the ligands and the oligonucleotides, and the optional use of linkers to facilitate such conjugation.

In one embodiment, the conjugate is a conjugate of an adaptor oligonucleotide and certain TLR7 specific ligands such as but not limited to immunosine or loxoribine. The adaptor oligonucleotide may be but is not limited to a poly(dT) oligonucleotide ranging in

length from 8-20, preferably 10-20, and more preferably 14-18 nucleotides in length. In some embodiments, conjugates of adaptor oligonucleotides and certain TLR7/8 ligands such as imidazoquinolines and C8-substituted guanosines are excluded, particularly if the adaptor oligonucleotide is itself immunostimulatory or signals through TLR9.

FIGs. 13A-C illustrate the structure of immunosine as well as two monomer variants thereof that can be used in the conjugation process. The variant shown in FIG. 13B can be conjugated to an oligonucleotide at an internal position or at a 3' end. Thus, there may be one or more of these variants attached to an oligonucleotide. The variant shown in FIG. 13C can be conjugated to an oligonucleotide at a 5' end. FIG. 14 illustrates the conjugation of immunosine to an oligonucleotide using a linker. FIG. 15 illustrates attachment points in R-848, CL-029 and immunosine to which linkers and/or oligonucleotides may be conjugated. The conjugates of the invention may comprise 1, 2, 3, 4, 5, or more linkers, or alternatively, they may lack linkers. An example of a conjugate is poly(dT)₁₄-L₂-IM (where L represents a linker and IM represents immunosine and subscripts denote the number of each unit in the conjugate) (SEQ ID NO: 9). Another example is IM-L₂-(dT)₁₄ (SEQ ID NO: 10). The difference between these examples is the placement of the immunosine and the linkers at either the 3' end or the 5' end.

The linkers may be attached to any reactive moiety on the oligonucleotide including but not limited to a backbone phosphate group or a sugar hydroxyl group. For example, they may be incorporated via phosphodiester, phosphorothioate, methylphosphonate and/or amide linkages.

The linkers may be non-nucleotide in nature. These include, for example, abasic residues (dSpacer), oligoethyleneglycol such as triethyleneglycol (spacer 9) or hexaethyleneglycol (spacer 18), or alkane-diol such as butanediol. Linkers may be attached to each other by phosphodiester or phosphorothioate bonds. Other linkers are alkylamino linkers, such as C3, C6, C12 amino linkers, and also alkylthiol linkers, such as C3 or C6 thiol linkers. Different types of linkers may also be used in one conjugate.

The oligonucleotides may be linked by aromatic residues which may be further substituted by alkyl or substituted alkyl groups. The oligonucleotides may also contain a doubler or trebler unit, which allow conjugation of multiple ligands of one or different types to the oligonucleotide. The oligonucleotides may also contain linker resulting from peptide modifying reagents or oligonucleotide modifying reagents. Furthermore, they may contain

one or more natural or unnatural amino acid residues which are connected by peptide (amide) linkages.

The different oligonucleotides are synthesized by established methods and can be linked together on-line during solid-phase synthesis. Alternatively, they may be linked together following synthesis of the individual units.

Immune Responses

In one embodiment, the TLR-mediated immune response is Th1-like immune response. As used herein, the term "Th1-like" refers to having a feature characteristic of a Th1 immune response. A Th1 immune response characteristically may include induction of certain cytokines such as IFN- γ , secretion (in mice) of IgG2a immunoglobulins, and macrophage activation. The term "Th1-like" is to be contrasted with the term "Th2-like", which refers to having a feature characteristic of a Th2 immune response, as discussed below.

A Th1-like immune response can include expression of any of certain cytokines and chemokines, including IFN- α , IFN- β , IFN- γ , TNF- α , IL-12, IL-18, IP-10, and any combination thereof, that are characteristically associated with a Th1 immune response. Th1 immune responses and Th2 immune responses are believed to be counter-regulatory. In some embodiments the Th1-like immune response can include suppression of certain Th2-associated cytokines, including IL-4, IL-5, IL-10, and IL-13. The Th1-like immune response can include expression of certain antibody isotypes, including (in the mouse) IgG2a, with or without suppression of certain Th2-associated antibody isotypes, including IgE and (in the mouse) IgG1. In one embodiment a Th1-like immune response is a Th1 response.

A Th2-like immune response can include expression of any of certain cytokines and chemokines, including IL-4, IL-5, IL-10, IL-13, and any combination thereof, that are characteristically associated with a Th2 immune response. In some embodiments the Th2-like immune response can include suppression of certain Th1-associated cytokines. The Th2-like immune response can include expression of certain antibody isotypes, including IgE and (in the mouse) IgG1, with or without suppression of certain Th1-associated antibody isotypes, including (in the mouse) IgG2a. In one embodiment a Th2-like immune response is a Th2 response.

Thus, in one embodiment the invention provides a method for inducing a Th1-like immune response in a subject. Inducing a Th1-like immune response includes augmenting or enhancing a Th1-like immune response. The method includes the step of administering to a

subject an effective amount of an TLR7/8 ligand and adaptor oligonucleotide of the invention to induce a Th1-like immune response in the subject.

In one embodiment the invention provides a method for suppressing a Th2-like immune response in a subject. The method includes the step of administering to a subject an effective amount of an TLR7/8 ligand and adaptor oligonucleotide of the invention to suppress a Th2-like immune response in the subject. Such method may find particular use in the treatment of subjects having or at risk of having a condition characterized by an immune response with predominant Th2 character. Such conditions include, without limitation, allergy and asthma.

In one embodiment the immune response involves upregulation of cell surface markers of immune cell activation, such as CD25, CD80, CD86, and CD154. Methods for measuring cell surface expression of such markers are well known in the art and include FACS analysis.

For measurement of immune response in a cell or population of cells, in one embodiment the cell or population of cells expresses at least one and in some instances preferably only one of TLR7, TLR8, or TLR9. The cell can express the TLR naturally, or it can be manipulated to express the TLR though introduction into the cell of a suitable expression vector for the TLR. In one embodiment the cell or population of cells is obtained as peripheral blood mononuclear cells (PBMC). In one embodiment the cell or population of cells is obtained as a cell line expressing the TLR. In one embodiment the cell or population of cells is obtained as a cell line transiently transfected with a TLR. In one embodiment the cell or population of cells is obtained as a cell line stably transfected with a TLR.

Also for use in measuring an immune response in a cell or population of cells, it may be convenient to introduce into the cell or population of cells a reporter construct that is responsive to (i.e., regulated by) intracellular signaling by a TLR. In one embodiment such a reporter is a gene placed under the control of an NF- κ B promoter. In one embodiment the gene placed under control of the promoter is luciferase although other reporter genes can be used including green fluorescent protein (GFP), β -galactosidase, alkaline phosphatase, and the like. Under suitable conditions of activation, as an example, the luciferase reporter construct is expressed and emits a detectable light signal that may be measured quantitatively using a luminometer. These and other reporter constructs are commercially available. In other embodiments, TLR-mediated immune responses can be measured by the production (mRNA or protein) or secretion of cytokines such as IFN-alpha, TNF-alpha, IL-12, IFN-gamma and

the like. The examples demonstrate these types of assays. The invention also contemplates the use of cell-free methods of detecting TLR activation.

The invention also includes a method for inducing antigen specific immune responses (as described in greater detail herein) and non-specific innate immune activation and broad spectrum resistance to infectious challenge. The term antigen non-specific innate immune activation, as used herein, refers to the activation of immune cells other than B cells including NK cells, T cells or other immune cells that can respond in an antigen independent fashion, or some combination of these cells. A broad spectrum resistance to infectious challenge is induced because the immune cells are in active form and are primed to respond to any invading compound or microorganism. The cells do not have to be specifically primed against a particular antigen. This is particularly useful in biowarfare, and the other circumstances described above such as travelers.

Subjects

As used herein, a subject refers to a human or non-human vertebrate. Non-human vertebrates include livestock animals, companion animals, and laboratory animals. Non-human subjects also specifically include non-human primates as well as rodents. Non-human subjects also specifically include, without limitation, chickens, horses, cows, pigs, goats, dogs, cats, guinea pigs, hamsters, mink, and rabbits.

As used herein, a subject at risk of developing a condition refers to a subject with a known or suspected exposure to an agent known to cause or to be associated with the condition or a known or suspected predisposition to develop the condition (e.g., a genetic marker for or a family history of the condition).

In one aspect the invention provides a method of treating a subject having an immune system deficiency. The method according to this aspect of the invention includes the step of administering to the subject an effective amount of a composition of the invention to treat the subject. An immune system deficiency as used herein refers to an abnormally depressed ability of an immune system to mount an immune response to an antigen. In one embodiment an immune system deficiency is a disease or disorder in which the subject's immune system is not functioning in normal capacity or in which it would be useful to boost the subject's immune response, for example to eliminate a tumor or cancer or an infection in the subject. A subject having an immune deficiency, as used herein, refers to a subject in whom there is a depressed ability of the subject's immune system to mount an immune response for example,

to an antigen. Subjects having an immune deficiency include subjects having an acquired immune deficiency as well as subjects having a congenital immune system deficiency. Subjects having acquired immune deficiency include, without limitation, subjects having a chronic inflammatory condition, subjects having chronic renal insufficiency or renal failure, subjects having infection, subjects having cancer, subjects receiving immunosuppressive drugs, subjects receiving other immunosuppressive treatment, and subjects with malnutrition. In one embodiment the subject has a suppressed CD4+ T-cell population. In one embodiment the subject has an infection with human immunodeficiency virus (HIV) or has acquired immunodeficiency syndrome (AIDS). The method according to this aspect of the invention thus provides a method for boosting an immune response or boosting the ability to mount an immune response in a subject in need of a more vigorous immune response.

As used herein, inhibit shall mean reduce an outcome or effect compared to normal.

As used herein, treat as used in reference to a disease or condition shall mean to intervene in such disease or condition so as to prevent or slow the development of, prevent or slow the progression of, halt the progression of, or eliminate the disease or condition. For example, treating cancer includes preventing the development of a cancer (e.g., from a precancerous state), reducing the symptoms of cancer, and/or inhibiting the growth of an established cancer.

Conditions

The invention intends to treat conditions which would benefit from inhibition and/or induction of certain TLR-mediated immune responses. As used herein, a condition associated with TLR7-mediated immunostimulation or immune response refers to any disease or other condition in a subject in which there is immune activation associated with TLR7 signaling, and such activation is detrimental. Such conditions typically involve activation of TLR7 signaling in response to contact with a TLR7 ligand.

As used herein, a condition associated with TLR8-mediated immunostimulation or immune response refers to any disease or other condition in a subject in which there is immune activation associated with TLR8 signaling, and such activation is detrimental. Such conditions typically involve activation of TLR8 signaling in response to contact with a TLR8 ligand.

As used herein, a condition associated with TLR9-mediated immunostimulation or immune response refers to any disease or other condition in a subject in which there is

immune activation associated with TLR9 signaling, and such activation is detrimental. Such conditions typically involve activation of TLR9 signaling in response to contact with a TLR9 ligand.

Infection

The invention can be used to treat conditions such as infection. Infection refers to any condition in which there is an abnormal collection or population of viable intracellular or extracellular microbes in a subject. Such microbes may be endogenous to the subject or they may be foreign to the subject. A subject having an infection is a subject that has a disorder arising from the invasion of the subject, superficially, locally, or systemically, by an infectious microorganism or from abnormal upregulated growth of naturally occurring endogenous microbes in a subject. The infectious microorganism can be a virus, bacterium, fungus, or parasite, as described above. Various types of microbes can cause infection, including microbes that are bacteria, microbes that are viruses, microbes that are fungi, and microbes that are parasites.

Bacteria are unicellular organisms which multiply asexually by binary fission. They are classified and named based on their morphology, staining reactions, nutrition and metabolic requirements, antigenic structure, chemical composition, and genetic homology. Bacteria can be classified into three groups based on their morphological forms, spherical (coccus), straight-rod (bacillus) and curved or spiral rod (vibrio, campylobacter, spirillum, and spirochaete). Bacteria are also more commonly characterized based on their staining reactions into two classes of organisms, gram-positive and gram-negative. Gram refers to the method of staining which is commonly performed in microbiology labs. Gram-positive organisms retain the stain following the staining procedure and appear a deep violet color. Gram-negative organisms do not retain the stain but take up the counter-stain and thus appear pink.

Infectious bacteria include, but are not limited to, gram negative and gram positive bacteria. Gram positive bacteria include, but are not limited to *Pasteurella* species, *Staphylococci* species, and *Streptococcus* species. Gram negative bacteria include, but are not limited to, *Escherichia coli*, *Pseudomonas* species, and *Salmonella* species. Specific examples of infectious bacteria include but are not limited to: *Helicobacter pyloris*, *Borrelia burgdorferi*, *Legionella pneumophila*, *Mycobacteria* spp (e.g., *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. gordonae*), *Staphylococcus aureus*, *Neisseria gonorrhoeae*,

Neisseria meningitidis, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A *Streptococcus*), *Streptococcus agalactiae* (Group B *Streptococcus*), *Streptococcus* (viridans group), *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus* (anaerobic species), *Streptococcus pneumoniae*, pathogenic *Campylobacter* sp., *Enterococcus* sp., *Haemophilus influenzae*, *Bacillus anthracis*, *Corynebacterium diphtheriae*, *Corynebacterium* sp., *Erysipelothrix rhusiopathiae*, *Clostridium perfringens*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Bacteroides* sp., *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidum*, *Treponema pertenue*, *Leptospira*, *Rickettsia*, and *Actinomyces israelii*.

Bacteria include, but are not limited to, *Pasteurella* species, *Staphylococci* species, *Streptococcus* species, *Escherichia coli*, *Pseudomonas* species, and *Salmonella* species. Specific examples of infectious bacteria include but are not limited to, *Helicobacter pyloris*, *Borrelia burgdorferi*, *Legionella pneumophila*, *Mycobacteria* sps (e.g., *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. gordonae*), *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A *Streptococcus*), *Streptococcus agalactiae* (Group B *Streptococcus*), *Streptococcus* (viridans group), *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus* (anaerobic sps.), *Streptococcus pneumoniae*, pathogenic *Campylobacter* sp., *Enterococcus* sp., *Haemophilus influenzae*, *Bacillus anthracis*, *Corynebacterium diphtheriae*, *Corynebacterium* sp., *Erysipelothrix rhusiopathiae*, *Clostridium perfringens*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Bacteroides* sp., *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidum*, *Treponema pertenue*, *Leptospira*, *Rickettsia*, and *Actinomyces israelii*.

Viruses are small infectious agents which generally contain a nucleic acid core and a protein coat, but are not independently living organisms. Viruses can also take the form of infectious nucleic acids lacking a protein. A virus cannot survive in the absence of a living cell within which it can replicate. Viruses enter specific living cells either by endocytosis or direct injection of DNA (phage) and multiply, causing disease. The multiplied virus can then be released and infect additional cells. Some viruses are DNA-containing viruses and others are RNA-containing viruses. In some aspects, the invention also intends to treat diseases in which prions are implicated in disease progression such as for example bovine spongiform encephalopathy (i.e., mad cow disease, BSE) or scrapie infection in animals, or Creutzfeldt-Jakob disease in humans.

Viruses include, but are not limited to, enteroviruses (including, but not limited to, viruses that the family *Picornaviridae*, such as polio virus, coxsackie virus, echo virus), rotaviruses, adenovirus, hepatitis virus. Specific examples of viruses that have been found in humans include but are not limited to: *Retroviridae* (e.g., human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HTV-LP; *Picornaviridae* (e.g., polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); *Calciviridae* (e.g., strains that cause gastroenteritis); *Togaviridae* (e.g., equine encephalitis viruses, rubella viruses); *Flaviviridae* (e.g., dengue viruses, encephalitis viruses, yellow fever viruses); *Coronaviridae* (e.g., coronaviruses); *Rhabdoviridae* (e.g., vesicular stomatitis viruses, rabies viruses); *Filoviridae* (e.g., ebola viruses); *Paramyxoviridae* (e.g., parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); *Orthomyxoviridae* (e.g., influenza viruses); *Bunyaviridae* (e.g., Hantaan viruses, bunya viruses, phleboviruses and Nairo viruses); *Arenaviridae* (hemorrhagic fever viruses); *Reoviridae* (e.g., reoviruses, orbiviruses and rotaviruses); *Birnaviridae*; *Hepadnaviridae* (Hepatitis B virus); *Parvoviridae* (parvoviruses); *Papovaviridae* (papillomaviruses, polyoma viruses); *Adenoviridae* (most adenoviruses); *Herpesviridae* (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV)); *Poxviridae* (variola viruses, vaccinia viruses, pox viruses); *Iridoviridae* (e.g., African swine fever virus); and unclassified viruses (e.g., the etiological agents of spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1 = internally transmitted; class 2 = parenterally transmitted (i.e., Hepatitis C); Norwalk and related viruses, and astroviruses).

Examples of viruses that have been found in humans include but are not limited to: *Retroviridae* (e.g., human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HTV-LP; *Picornaviridae* (e.g., polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); *Calciviridae* (e.g., strains that cause gastroenteritis); *Togaviridae* (e.g., equine encephalitis viruses, rubella viruses); *Flaviviridae* (e.g., dengue viruses, encephalitis viruses, yellow fever viruses); *Coronaviridae* (e.g., coronaviruses); *Rhabdoviridae* (e.g., vesicular stomatitis viruses, rabies viruses); *Filoviridae* (e.g., ebola viruses); *Paramyxoviridae* (e.g., parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); *Orthomyxoviridae* (e.g., influenza viruses); *Bunyaviridae* (e.g., Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); *Arenaviridae* (hemorrhagic

fever viruses); *Reoviridae* (e.g., reoviruses, orbiviruses and rotaviruses); *Bornaviridae*; *Hepadnaviridae* (Hepatitis B virus); *Parvoviridae* (parvoviruses); *Papovaviridae* (papilloma viruses, polyoma viruses); *Adenoviridae* (most adenoviruses); *Herpesviridae* (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes virus; *Poxviridae* (variola viruses, vaccinia viruses, pox viruses); and *Iridoviridae* (e.g., African swine fever virus); and unclassified viruses (e.g., the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), Hepatitis C; Norwalk and related viruses, and astroviruses).

Fungi are eukaryotic organisms, only a few of which cause infection in vertebrate mammals. Because fungi are eukaryotic organisms, they differ significantly from prokaryotic bacteria in size, structural organization, life cycle and mechanism of multiplication. Fungi are classified generally based on morphological features, modes of reproduction and culture characteristics. Although fungi can cause different types of disease in subjects, such as respiratory allergies following inhalation of fungal antigens, fungal intoxication due to ingestion of toxic substances, such as *Amanita phalloides* toxin and phallotoxin produced by poisonous mushrooms and aflatoxins, produced by aspergillus species, not all fungi cause infectious disease.

Infectious fungi can cause systemic or superficial infections. Primary systemic infection can occur in normal healthy subjects, and opportunistic infections are most frequently found in immunocompromised subjects. The most common fungal agents causing primary systemic infection include *Blastomyces*, *Coccidioides*, and *Histoplasma*. Common fungi causing opportunistic infection in immunocompromised or immunosuppressed subjects include, but are not limited to, *Candida albicans*, *Cryptococcus neoformans*, and various *Aspergillus* species. Systemic fungal infections are invasive infections of the internal organs. The organism usually enters the body through the lungs, gastrointestinal tract, or intravenous catheters. These types of infections can be caused by primary pathogenic fungi or opportunistic fungi.

Superficial fungal infections involve growth of fungi on an external surface without invasion of internal tissues. Typical superficial fungal infections include cutaneous fungal infections involving skin, hair, or nails.

Diseases associated with fungal infection include aspergillosis, blastomycosis, candidiasis, chromoblastomycosis, coccidioidomycosis, cryptococcosis, fungal eye infections, fungal hair, nail, and skin infections, histoplasmosis, lobomycosis, mycetoma, otomycosis,

paracoccidioidomycosis, disseminated *Penicillium marneffei*, phaeohyphomycosis, rhinosporidiosis, sporotrichosis, and zygomycosis.

Fungi include yeasts and molds. Examples of fungi include without limitation *Aspergillus* spp including *Aspergillus fumigatus*, *Blastomyces dermatitidis*, *Candida* spp including *Candida albicans*, *Coccidioides immitis*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Pneumocystis carinii*, *Rhizomucor* spp, and *Rhizopus* spp.

Parasites are organisms which depend upon other organisms in order to survive and thus must enter, or infect, another organism to continue their life cycle. The infected organism, i.e., the host, provides both nutrition and habitat to the parasite. Although in its broadest sense the term parasite can include all infectious agents (i.e., bacteria, viruses, fungi, protozoa and helminths), generally speaking, the term is used to refer solely to protozoa, helminths, and ectoparasitic arthropods (e.g., ticks, mites, etc.). Protozoa are single-celled organisms which can replicate both intracellularly and extracellularly, particularly in the blood, intestinal tract or the extracellular matrix of tissues. Helminths are multicellular organisms which almost always are extracellular (an exception being *Trichinella* spp.). Helminths normally require exit from a primary host and transmission into a secondary host in order to replicate. In contrast to these aforementioned classes, ectoparasitic arthropods form a parasitic relationship with the external surface of the host body.

Parasites include intracellular parasites and obligate intracellular parasites. Examples of parasites include but are not limited to *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium vivax*, *Plasmodium knowlesi*, *Babesia microti*, *Babesia divergens*, *Trypanosoma cruzi*, *Toxoplasma gondii*, *Trichinella spiralis*, *Leishmania major*, *Leishmania donovani*, *Leishmania braziliensis*, *Leishmania tropica*, *Trypanosoma gambiense*, *Trypanosoma rhodesiense* and *Schistosoma mansoni*.

Other infectious organisms (i.e., protists) include *Plasmodium* spp. such as *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium vivax* and *Toxoplasma gondii*. Blood-borne and/or tissue parasites include *Plasmodium* spp., *Babesia microti*, *Babesia divergens*, *Chlamydia trachomatis*, *Leishmania tropica*, *Leishmania* spp., *Leishmania braziliensis*, *Leishmania donovani*, *Trypanosoma gambiense* and *Trypanosoma rhodesiense* (African sleeping sickness), *Trypanosoma cruzi* (Chagas' disease), and *Toxoplasma gondii*.

Other medically relevant microorganisms have been described extensively in the literature, e.g., see C.G.A Thomas, *Medical Microbiology*, Bailliere Tindall, Great Britain 1983, the entire contents of which is hereby incorporated by reference.

Cancer

In one aspect the invention provides a method of treating a subject having a cancer.

A subject having a cancer is a subject that has detectable cancerous cells. The cancer may be a malignant or non-malignant cancer. "Cancer" as used herein refers to an uncontrolled growth of cells which interferes with the normal functioning of the bodily organs and systems. Cancers which migrate from their original location and seed vital organs can eventually lead to the death of the subject through the functional deterioration of the affected organs. Hemopoietic cancers, such as leukemia, are able to outcompete the normal hemopoietic compartments in a subject, thereby leading to hemopoietic failure (in the form of anemia, thrombocytopenia and neutropenia) ultimately causing death.

A metastasis is a region of cancer cells, distinct from the primary tumor location, resulting from the dissemination of cancer cells from the primary tumor to other parts of the body. At the time of diagnosis of the primary tumor mass, the subject may be monitored for the presence of metastases. Metastases are most often detected through the sole or combined use of magnetic resonance imaging (MRI) scans, computed tomography (CT) scans, blood and platelet counts, liver function studies, chest X-rays and bone scans in addition to the monitoring of specific symptoms.

Cancers include, but are not limited to, basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and central nervous system (CNS) cancer; breast cancer; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; intra-epithelial neoplasm; kidney cancer; larynx cancer; leukemia; liver cancer; lung cancer (e.g., small cell and non-small cell); lymphoma including Hodgkin's and Non-Hodgkin's lymphoma; melanoma; myeloma; neuroblastoma; oral cavity cancer (e.g., lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; sarcoma; skin cancer; stomach cancer; testicular cancer; thyroid cancer; uterine cancer; cancer of the urinary system, as well as other carcinomas, adenocarcinomas, and sarcomas.

Autoimmune Conditions

Conditions that involve an innate immune response or a Th1-like immune response, include inflammation, acute and chronic allograft rejection, graft-versus-host disease (GvHD), certain autoimmune diseases, and sepsis. The invention can be used to treat such conditions in view of the selective inhibition of TLR signaling that can be achieved according to the invention.

Autoimmune diseases can be generally classified as antibody-mediated, T-cell mediated, or a combination of antibody-mediated and T-cell mediated. The adaptor ODN and TLR ligand combinations of the invention are believed to be useful for treating various types of autoimmunity involving antibody-mediated or T-cell mediated immunity, including insulin-dependent (type I) diabetes mellitus, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus (SLE), and inflammatory bowel disease (i.e., Crohn's disease and ulcerative colitis). Animal models for these autoimmune diseases are available and are useful for assessing the efficacy of the combinations of the invention in these diseases. Other autoimmune diseases include, without limitation, alopecia areata, acquired hemophilia, ankylosing spondylitis, antiphospholipid syndrome, autoimmune hepatitis, autoimmune hemolytic anemia, Behcet's syndrome, cardiomyopathy, celiac sprue dermatitis, chronic fatigue immune dysfunction syndrome (CFIDS), chronic inflammatory demyelinating polyneuropathy, Churg-Strauss syndrome, cicatricial pemphigoid, CREST syndrome, cold agglutinin disease, discoid lupus, essential mixed cryoglobulinemia, fibromyalgia, fibromyositis, Guillain-Barré syndrome, idiopathic pulmonary fibrosis, idiopathic thrombocytopenic purpura, IgA nephropathy, juvenile arthritis, lichen planus, myasthenia gravis, polyarteritis nodosa, polychondritis, polyglandular syndromes, dermatomyositis, primary agammaglobulinemia, primary biliary cirrhosis, psoriasis, Raynaud's phenomena, Reiter's syndrome, sarcoidosis, stiff-man syndrome, Takayasu arthritis, temporal arteritis/giant cell arteritis, uveitis, vasculitis, and vitiligo.

In several autoimmune diseases antibodies to self antigens are frequently observed. For example for systemic lupus erythematosus autoantibodies have been described to single-stranded and double-stranded DNA or RNA. Vallin H et al. (1999) *J Immunol* 163:6306-13; Hoet RM et al. (1999) *J Immunol* 163:3304-12; ven Venrooij (1990) *J Clin Invest* 86:2154-60. The levels of autoantibodies found in the serum of autoimmune patients very often are found to correlate with disease severity. The pattern of autoantibodies that arise, e.g., in human SLE, suggest that intact macromolecular particles, such as RNA- or DNA-containing

complexes, could themselves be immunogenic and anti-nucleic acid antibodies could therefore arise. Lotz M et al. (1992) *Mol Biol Rep* 16:127; Mohan C et al. (1993) *J Exp Med* 177:1367-81. Such DNA or RNA released from, e.g., apoptotic cells or DNA- or RNA-containing microbes present in serum of autoimmune patients, could be responsible for inflammation that contributes to the autoimmune disease. Fatenejad S (1994) *J Immunol* 152:5523-31; Malmegrim KC et al. (2002) *Isr Med Assoc J* 4:706-12; Newkirk MM et al. (2001) *Arthritis Res* 3:253-8. Indeed CpG-containing sequences could be identified from SLE serum that induces an efficient immune response dominated by IFN- α secretion that is thought to contribute to the development of autoimmune diseases. Magnusson M et al. (2001) *Scand J Immunol* 54:543-50; Rönnblom L et al. (2001) *J Exp Med* 194:F59-63. In addition, the epitopes for anti-RNA antibodies could be identified and are composed of G,U-rich sequences. Tsai DE et al. (1992) *Proc Natl Acad Sci USA* 89:8864-8; Tsai DE et al. (1993) *J Immunol* 150:1137-45.

Allergy

An "allergic condition" or "allergy" refers to acquired hypersensitivity to a substance (allergen). A "subject having an allergic condition" shall refer to a subject that is currently experiencing or has previously experienced an allergic reaction in response to an allergen. Allergic conditions include but are not limited to eczema, allergic rhinitis or coryza, hay fever, allergic conjunctivitis, bronchial asthma, urticaria (hives) and food allergies, other atopic conditions including atopic dermatitis; anaphylaxis; drug allergy; and angioedema.

Allergy is typically an episodic condition associated with the production of antibodies from a particular class of immunoglobulin, IgE, against allergens. The development of an IgE-mediated response to common aeroallergens is also a factor which indicates predisposition towards the development of asthma. If an allergen encounters a specific IgE which is bound to an IgE Fc receptor (Fc ϵ R) on the surface of a basophil (circulating in the blood) or mast cell (dispersed throughout solid tissue), the cell becomes activated, resulting in the production and release of mediators such as histamine, serotonin, and lipid mediators.

An allergic reaction occurs when tissue-sensitizing immunoglobulin of the IgE type reacts with foreign allergen. The IgE antibody is bound to mast cells and/or basophils, and these specialized cells release chemical mediators (vasoactive amines) of the allergic reaction when stimulated to do so by allergens bridging the ends of the antibody molecule. Histamine, platelet activating factor, arachidonic acid metabolites, and serotonin are among the best

known mediators of allergic reactions in man. Histamine and the other vasoactive amines are normally stored in mast cells and basophil leukocytes. The mast cells are dispersed throughout animal tissue and the basophils circulate within the vascular system. These cells manufacture and store histamine within the cell unless the specialized sequence of events involving IgE binding occurs to trigger its release.

Symptoms of an allergic reaction vary, depending on the location within the body where the IgE reacts with the antigen. If the reaction occurs along the respiratory epithelium, the symptoms generally are sneezing, coughing and asthmatic reactions. If the interaction occurs in the digestive tract, as in the case of food allergies, abdominal pain and diarrhea are common. Systemic allergic reactions, for example following a bee sting or administration of penicillin to an allergic subject, can be severe and often life-threatening.

Allergy is associated with a Th2-type of immune response, which is characterized at least in part by Th2 cytokines IL-4 and IL-5, as well as antibody isotype switching to IgE. Th1 and Th2 immune responses are mutually counter-regulatory, so that skewing of the immune response toward a Th1-type of immune response can prevent or ameliorate a Th2-type of immune response, including allergy.

Asthma

“Asthma” as used herein refers to a disorder of the respiratory system characterized by inflammation and narrowing of the airways, and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively, associated with an atopic or allergic condition. Symptoms of asthma include recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, resulting from airflow obstruction. Airway inflammation associated with asthma can be detected through observation of a number of physiological changes, such as, denudation of airway epithelium, collagen deposition beneath basement membrane, edema, mast cell activation, inflammatory cell infiltration, including neutrophils, eosinophils, and lymphocytes. As a result of the airway inflammation, asthma patients often experience airway hyper-responsiveness, airflow limitation, respiratory symptoms, and disease chronicity. Airflow limitations include acute bronchoconstriction, airway edema, mucous plug formation, and airway remodeling, features which often lead to bronchial obstruction. In some cases of asthma, sub-basement membrane fibrosis may occur, leading to persistent abnormalities in lung function.

Research over the past several years has revealed that asthma likely results from complex interactions among inflammatory cells, mediators, and other cells and tissues resident in the airways. Mast cells, eosinophils, epithelial cells, macrophage, and activated T cells all play an important role in the inflammatory process associated with asthma. Djukanovic R et al. (1990) *Am Rev Respir Dis* 142:434-457. It is believed that these cells can influence airway function through secretion of preformed and newly synthesized mediators which can act directly or indirectly on the local tissue. It has also been recognized that subpopulations of T lymphocytes (Th2) play an important role in regulating allergic inflammation in the airway by releasing selective cytokines and establishing disease chronicity. Robinson DS et al. (1992) *N Engl J Med* 326:298-304.

Asthma is a complex disorder which arises at different stages in development and can be classified based on the degree of symptoms as acute, subacute, or chronic. An acute inflammatory response is associated with an early recruitment of cells into the airway. The subacute inflammatory response involves the recruitment of cells as well as the activation of resident cells causing a more persistent pattern of inflammation. Chronic inflammatory response is characterized by a persistent level of cell damage and an ongoing repair process, which may result in permanent abnormalities in the airway.

A "subject having asthma" is a subject that has a disorder of the respiratory system characterized by inflammation and narrowing of the airways and increased reactivity of the airways to inhaled agents. Factors associated with initiation of asthma include, but are not limited to, allergens, cold temperature, exercise, viral infections, and SO₂.

As mentioned herein, asthma may be associated with a Th2-type of immune response, which is characterized at least in part by Th2 cytokines IL-4 and IL-5, as well as antibody isotype switching to IgE. Th1 and Th2 immune responses are mutually counter-regulatory, so that skewing of the immune response toward a Th1-type of immune response can prevent or ameliorate a Th2-type of immune response, including allergy.

The TLR7/8 ligand and adaptor oligonucleotide combination of the invention is also useful for improving survival, differentiation, activation and maturation of dendritic cells.

In certain aspects the invention provides a method for enhancing epitope spreading. "Epitope spreading" as used herein refers to the diversification of epitope specificity from an initial focused, dominant epitope-specific immune response, directed against a self or foreign

protein, to subdominant and/or cryptic epitopes on that protein (intramolecular spreading) or other proteins (intermolecular spreading). Epitope spreading results in multiple epitope-specific immune responses.

Anti-microbial agents

The TLR7/8 ligand and adaptor oligonucleotide combination can be used together with one or more anti-microbial agents. Anti-microbial agents or medicaments include but are not limited to anti-bacterial agents, anti-viral agents, anti-fungal agents and anti-parasitic agents. Phrases such as "anti-infective agent", "antibiotic", "anti-bacterial agent", "anti-viral agent", "anti-fungal agent", "anti-parasitic agent" and "parasiticide" have well-established meanings to those of ordinary skill in the art and are defined in standard medical texts. Briefly, anti-bacterial agents kill or inhibit bacteria, and include antibiotics as well as other synthetic or natural compounds having similar functions. Anti-viral agents can be isolated from natural sources or synthesized and are useful for killing or inhibiting viruses. Anti-fungal agents are used to treat superficial fungal infections as well as opportunistic and primary systemic fungal infections. Anti-parasite agents kill or inhibit parasites. Many antibiotics are low molecular weight molecules which are produced as secondary metabolites by cells, such as microorganisms. In general, antibiotics interfere with one or more functions or structures which are specific for the microorganism and which are not present in host cells.

One of the problems with anti-infective therapies is the side effects occurring in the host that is treated with the anti-infective agent. For instance, many anti-infectious agents can kill or inhibit a broad spectrum of microorganisms and are not specific for a particular type of species. Treatment with these types of anti-infectious agents results in the killing of the normal microbial flora living in the host, as well as the infectious microorganism. The loss of the microbial flora can lead to disease complications and predispose the host to infection by other pathogens, since the microbial flora compete with and function as barriers to infectious pathogens. Other side effects may arise as a result of specific or non-specific effects of these chemical entities on non-microbial cells or tissues of the host.

Another problem with widespread use of anti-infectants is the development of antibiotic-resistant strains of microorganisms. Already, vancomycin-resistant *enterococci*, penicillin-resistant *pneumococci*, multi-resistant *S. aureus*, and multi-resistant *tuberculosis* strains have developed and are becoming major clinical problems. Widespread use of anti-

infectants will likely produce many antibiotic-resistant strains of bacteria. As a result, new anti-infective strategies will be required to combat these microorganisms.

Anti-bacterial antibiotics which are effective for killing or inhibiting a wide range of bacteria are referred to as broad-spectrum antibiotics. Other types of antibacterial antibiotics are predominantly effective against the bacteria of the class gram-positive or gram-negative. These types of antibiotics are referred to as narrow-spectrum antibiotics. Other antibiotics which are effective against a single organism or disease and not against other types of bacteria, are referred to as limited-spectrum antibiotics.

Anti-bacterial agents are sometimes classified based on their primary mode of action. In general, anti-bacterial agents are cell wall synthesis inhibitors, cell membrane inhibitors, protein synthesis inhibitors, nucleic acid synthesis or functional inhibitors, and competitive inhibitors. Cell wall synthesis inhibitors inhibit a step in the process of cell wall synthesis, and in general in the synthesis of bacterial peptidoglycan. Cell wall synthesis inhibitors include β -lactam antibiotics, natural penicillins, semi-synthetic penicillins, ampicillin, clavulanic acid, cephalosporins, and bacitracin.

The β -lactams are antibiotics containing a four-membered β -lactam ring which inhibits the last step of peptidoglycan synthesis. β -lactam antibiotics can be synthesized or natural. The β -lactam antibiotics produced by *penicillium* are the natural penicillins, such as penicillin G or penicillin V. These are produced by fermentation of *Penicillium chrysogenum*. The natural penicillins have a narrow spectrum of activity and are generally effective against *Streptococcus*, *Gonococcus*, and *Staphylococcus*. Other types of natural penicillins, which are also effective against gram-positive bacteria, include penicillins F, X, K, and O.

Semi-synthetic penicillins are generally modifications of the molecule 6-aminopenicillanic acid produced by a mold. The 6-aminopenicillanic acid can be modified by addition of side chains which produce penicillins having broader spectrums of activity than natural penicillins or various other advantageous properties. Some types of semi-synthetic penicillins have broad spectrums against gram-positive and gram-negative bacteria, but are inactivated by penicillinase. These semi-synthetic penicillins include ampicillin, carbenicillin, oxacillin, azlocillin, mezlocillin, and piperacillin. Other types of semi-synthetic penicillins have narrower activities against gram-positive bacteria, but have developed properties such that they are not inactivated by penicillinase. These include, for instance, methicillin, dicloxacillin, and nafcillin. Some of the broad spectrum semi-synthetic penicillins can be used in combination with β -lactamase inhibitors, such as clavulanic acids

and sulbactam. The β -lactamase inhibitors do not have anti-microbial action but they function to inhibit penicillinase, thus protecting the semi-synthetic penicillin from degradation.

Another type of β -lactam antibiotic is the cephalosporins. They are sensitive to degradation by bacterial β -lactamases, and thus, are not always effective alone. Cephalosporins, however, are resistant to penicillinase. They are effective against a variety of gram-positive and gram-negative bacteria. Cephalosporins include, but are not limited to, cephalexin, cefaclor, cefazolin, cefuroxine, cefoxitin, cefotaxime, cefsulodin, cefetamet, cefixime, ceftriaxone, cefoperazone, ceftazidine, and moxalactam.

Bacitracin is another class of antibiotics which inhibit cell wall synthesis, by inhibiting the release of muropeptide subunits or peptidoglycan from the molecule that delivers the subunit to the outside of the membrane. Although bacitracin is effective against gram-positive bacteria, its use is limited in general to topical administration because of its high toxicity.

Carbapenems are another broad-spectrum β -lactam antibiotic, which is capable of inhibiting cell wall synthesis. Examples of carbapenems include, but are not limited to, imipenems. Monobactams are also broad-spectrum β -lactam antibiotics, and include, euztreonam. An antibiotic produced by *Streptomyces*, vancomycin, is also effective against gram-positive bacteria by inhibiting cell membrane synthesis.

Another class of anti-bacterial agents is the anti-bacterial agents that are cell membrane inhibitors. These compounds disorganize the structure or inhibit the function of bacterial membranes. One problem with anti-bacterial agents that are cell membrane inhibitors is that they can produce effects in eukaryotic cells as well as bacteria because of the similarities in phospholipids in bacterial and eukaryotic membranes. Thus these compounds are rarely specific enough to permit these compounds to be used systemically and prevent the use of high doses for local administration.

One clinically useful cell membrane inhibitor is Polymyxin. Polymyxins interfere with membrane function by binding to membrane phospholipids. Polymyxin is effective mainly against Gram-negative bacteria and is generally used in severe *Pseudomonas* infections or *Pseudomonas* infections that are resistant to less toxic antibiotics. The severe side effects associated with systemic administration of this compound include damage to the kidney and other organs.

Other cell membrane inhibitors include Amphotericin B and Nystatin which are anti-fungal agents used predominantly in the treatment of systemic fungal infections and *Candida* yeast infections. Imidazoles are another class of antibiotic that is a cell membrane inhibitor. Imidazoles are used as anti-bacterial agents as well as anti-fungal agents, e.g., used for treatment of yeast infections, dermatophytic infections, and systemic fungal infections. Imidazoles include but are not limited to clotrimazole, miconazole, ketoconazole, itraconazole, and fluconazole.

Many anti-bacterial agents are protein synthesis inhibitors. These compounds prevent bacteria from synthesizing structural proteins and enzymes and thus cause inhibition of bacterial cell growth or function or cell death. In general these compounds interfere with the processes of transcription or translation. Anti-bacterial agents that block transcription include but are not limited to Rifampins and Ethambutol. Rifampins, which inhibit the enzyme RNA polymerase, have a broad spectrum activity and are effective against gram-positive and gram-negative bacteria as well as *Mycobacterium tuberculosis*. Ethambutol is effective against *Mycobacterium tuberculosis*.

Anti-bacterial agents which block translation interfere with bacterial ribosomes to prevent mRNA from being translated into proteins. In general this class of compounds includes but is not limited to tetracyclines, chloramphenicol, the macrolides (e.g., erythromycin) and the aminoglycosides (e.g., streptomycin).

The aminoglycosides are a class of antibiotics which are produced by the bacterium *Streptomyces*, such as, for instance streptomycin, kanamycin, tobramycin, amikacin, and gentamicin. Aminoglycosides have been used against a wide variety of bacterial infections caused by Gram-positive and Gram-negative bacteria. Streptomycin has been used extensively as a primary drug in the treatment of *tuberculosis*. Gentamicin is used against many strains of Gram-positive and Gram-negative bacteria, including *Pseudomonas* infections, especially in combination with Tobramycin. Kanamycin is used against many Gram-positive bacteria, including penicillin-resistant *Staphylococci*. One side effect of aminoglycosides that has limited their use clinically is that at dosages which are essential for efficacy, prolonged use has been shown to impair kidney function and cause damage to the auditory nerves leading to deafness.

Another type of translation inhibitor anti-bacterial agent is the tetracyclines. The tetracyclines are a class of antibiotics that are broad-spectrum and are effective against a variety of gram-positive and gram-negative bacteria. Examples of tetracyclines include

tetracycline, minocycline, doxycycline, and chlortetracycline. They are important for the treatment of many types of bacteria but are particularly important in the treatment of Lyme disease. As a result of their low toxicity and minimal direct side effects, the tetracyclines have been overused and misused by the medical community, leading to problems. For instance, their overuse has led to widespread development of resistance.

Anti-bacterial agents such as the macrolides bind reversibly to the 50 S ribosomal subunit and inhibit elongation of the protein by peptidyl transferase or prevent the release of uncharged tRNA from the bacterial ribosome or both. These compounds include erythromycin, roxithromycin, clarithromycin, oleandomycin, and azithromycin. Erythromycin is active against most Gram-positive bacteria, *Neisseria*, *Legionella* and *Haemophilus*, but not against the *Enterobacteriaceae*. Lincomycin and clindamycin, which block peptide bond formation during protein synthesis, are used against gram-positive bacteria.

Another type of translation inhibitor is chloramphenicol. Chloramphenicol binds the 70 S ribosome inhibiting the bacterial enzyme peptidyl transferase thereby preventing the growth of the polypeptide chain during protein synthesis. One serious side effect associated with chloramphenicol is aplastic anemia. Aplastic anemia develops at doses of chloramphenicol which are effective for treating bacteria in a small proportion (1/50,000) of patients. Chloramphenicol which was once a highly prescribed antibiotic is now seldom used as a result of the deaths from anemia. Because of its effectiveness it is still used in life-threatening situations (e.g., typhoid fever).

Some anti-bacterial agents disrupt nucleic acid synthesis or function, e.g., bind to DNA or RNA so that their messages cannot be read. These include but are not limited to quinolones and co-trimoxazole, both synthetic chemicals and rifamycins, a natural or semi-synthetic chemical. The quinolones block bacterial DNA replication by inhibiting the DNA gyrase, the enzyme needed by bacteria to produce their circular DNA. They are broad spectrum and examples include norfloxacin, ciprofloxacin, enoxacin, nalidixic acid and temafloxacin. Nalidixic acid is a bactericidal agent that binds to the DNA gyrase enzyme (topoisomerase) which is essential for DNA replication and allows supercoils to be relaxed and reformed, inhibiting DNA gyrase activity. The main use of nalidixic acid is in treatment of lower urinary tract infections (UTI) because it is effective against several types of Gram-negative bacteria such as *E. coli*, *Enterobacter aerogenes*, *K. pneumoniae* and *Proteus* species which are common causes of UTI. Co-trimoxazole is a combination of

sulfamethoxazole and trimethoprim, which blocks the bacterial synthesis of folic acid needed to make DNA nucleotides. Rifampicin is a derivative of rifamycin that is active against Gram-positive bacteria (including *Mycobacterium tuberculosis* and meningitis caused by *Neisseria meningitidis*) and some Gram-negative bacteria. Rifampicin binds to the beta subunit of the polymerase and blocks the addition of the first nucleotide which is necessary to activate the polymerase, thereby blocking mRNA synthesis.

Another class of anti-bacterial agents is compounds that function as competitive inhibitors of bacterial enzymes. The competitive inhibitors are mostly all structurally similar to a bacterial growth factor and compete for binding but do not perform the metabolic function in the cell. These compounds include sulfonamides and chemically modified forms of sulfanilamide which have even higher and broader antibacterial activity. The sulfonamides (e.g., gantrisin and trimethoprim) are useful for the treatment of *Streptococcus pneumoniae*, beta-hemolytic *streptococci* and *E. coli*, and have been used in the treatment of uncomplicated UTI caused by *E. coli*, and in the treatment of meningococcal meningitis.

Anti-viral agents are compounds which prevent infection of cells by viruses or replication of the virus within the cell. There are many fewer antiviral drugs than antibacterial drugs because the process of viral replication is so closely related to DNA replication within the host cell, that non-specific antiviral agents would often be toxic to the host. There are several stages within the process of viral infection which can be blocked or inhibited by antiviral agents. These stages include, attachment of the virus to the host cell (immunoglobulin or binding peptides), uncoating of the virus (e.g., amantadine), synthesis or translation of viral mRNA (e.g., interferon), replication of viral RNA or DNA (e.g., nucleoside analogues), maturation of new virus proteins (e.g., protease inhibitors), and budding and release of the virus.

Another category of anti-viral agents are nucleoside analogues. Nucleoside analogues are synthetic compounds which are similar to nucleosides, but which have an incomplete or abnormal deoxyribose or ribose group. Once the nucleoside analogues are in the cell, they are phosphorylated, producing the triphosphate form which competes with normal nucleotides for incorporation into the viral DNA or RNA. Once the triphosphate form of the nucleoside analogue is incorporated into the growing nucleic acid chain, it causes irreversible association with the viral polymerase and thus chain termination. Nucleoside analogues include, but are not limited to, acyclovir (used for the treatment of herpes simplex virus and varicella-zoster virus), gancyclovir (useful for the treatment of cytomegalovirus), idoxuridine, ribavirin

(useful for the treatment of respiratory syncitial virus), dideoxyinosine, dideoxycytidine, and zidovudine (azidothymidine).

Another class of anti-viral agents includes cytokines such as interferons. The interferons are cytokines which are secreted by virus-infected cells as well as immune cells. The interferons function by binding to specific receptors on cells adjacent to the infected cells, causing the change in the cell which protects it from infection by the virus. α and β -interferon also induce the expression of Class I and Class II MHC molecules on the surface of infected cells, resulting in increased antigen presentation for host immune cell recognition. α and β -interferons are available as recombinant forms and have been used for the treatment of chronic hepatitis B and C infection. At the dosages which are effective for anti-viral therapy, interferons have severe side effects such as fever, malaise and weight loss.

Immunoglobulin therapy is used for the prevention of viral infection. Immunoglobulin therapy for viral infections is different from bacterial infections, because rather than being antigen-specific, the immunoglobulin therapy functions by binding to extracellular virions and preventing them from attaching to and entering cells which are susceptible to the viral infection. The therapy is useful for the prevention of viral infection for the period of time that the antibodies are present in the host. In general there are two types of immunoglobulin therapies, normal immune globulin therapy and hyper-immune globulin therapy. Normal immune globulin therapy utilizes a antibody product which is prepared from the serum of normal blood donors and pooled. This pooled product contains low titers of antibody to a wide range of human viruses, such as hepatitis A, parvovirus, enterovirus (especially in neonates). Hyper-immune globulin therapy utilizes antibodies which are prepared from the serum of individuals who have high titers of an antibody to a particular virus. Those antibodies are then used against a specific virus. Examples of hyper-immune globulins include zoster immune globulin (useful for the prevention of varicella in immunocompromised children and neonates), human rabies immune globulin (useful in the post-exposure prophylaxis of a subject bitten by a rabid animal), hepatitis B immune globulin (useful in the prevention of hepatitis B virus, especially in a subject exposed to the virus), and RSV immune globulin (useful in the treatment of respiratory syncitial virus infections).

Anti-fungal agents are useful for the treatment and prevention of infective fungi. Anti-fungal agents are sometimes classified by their mechanism of action. Some anti-fungal agents function as cell wall inhibitors by inhibiting glucose synthase. These include, but are not limited to, basiungin/ECB. Other anti-fungal agents function by destabilizing membrane

integrity. These include, but are not limited to, imidazoles, such as clotrimazole, sertaconazole, fluconazole, itraconazole, ketoconazole, miconazole, and voriconazole, as well as FK 463, amphotericin B, BAY 38-9502, MK 991, pradimicin, UK 292, butenafine, and terbinafine. Other anti-fungal agents function by breaking down chitin (e.g., chitinase) or immunosuppression (501 cream).

Parasiticides are agents that kill parasites directly. Such compounds are known in the art and are generally commercially available. Examples of parasiticides useful for human administration include but are not limited to albendazole, amphotericin B, benznidazole, bithionol, chloroquine HCl, chloroquine phosphate, clindamycin, dehydroemetine, diethylcarbamazine, diloxanide furoate, eflornithine, furazolidone, glucocorticoids, halofantrine, iodoquinol, ivermectin, mebendazole, mefloquine, meglumine antimoniate, melarsoprol, metrifonate, metronidazole, niclosamide, nifurtimox, oxamniquine, paromomycin, pentamidine isethionate, piperazine, praziquantel, primaquine phosphate, proguanil, pyrantel pamoate, pyrimethamine-sulfonamides, pyrimethamine-sulfadoxine, quinacrine HCl, quinine sulfate, quinidine gluconate, spiramycin, stibogluconate sodium (sodium antimony gluconate), suramin, tetracycline, doxycycline, thiabendazole, tinidazole, trimethroprim-sulfamethoxazole, and tryparsamide.

Anti-cancer therapy

The TLR7/8 ligand and adaptor oligonucleotide combination of the invention can be used in conjunction with anti-cancer therapies.

Anti-cancer therapies include cancer medicaments, radiation, and surgical procedures. As used herein, a "cancer medicament" refers to an agent which is administered to a subject for the purpose of treating a cancer. Various types of medicaments for the treatment of cancer are described herein. For the purpose of this specification, cancer medicaments are classified as chemotherapeutic agents, immunotherapeutic agents, cancer vaccines, hormone therapy, and biological response modifiers.

The chemotherapeutic agent may be selected from the group consisting of methotrexate, vincristine, adriamycin, cisplatin, non-sugar containing chloroethylnitrosoureas, 5-fluorouracil, mitomycin C, bleomycin, doxorubicin, dacarbazine, taxol, fragyline, Meglamine GLA, valrubicin, carmustaine and poliferposan, MMI270, BAY 12-9566, RAS famesyl transferase inhibitor, famesyl transferase inhibitor, MMP, MTA/LY231514, Alimta, LY264618/Lometexol, Glamolec, CI-994, TNP-470, Hycamtin/Topotecan, PKC412,

Valspodar/PSC833, Novantrone/Mitroxantrone, Metaret/Suramin, Batimastat, E7070, BCH-4556, CS-682, 9-AC, AG3340, AG3433, Incel/VX-710, VX-853, ZD0101, ISI641, ODN 698, TA 2516/Marmistat, BB2516/Marmistat, CDP 845, D2163, PD183805, DX8951f, Lemonal DP 2202, FK 317, Picibanil/OK-432, AD 32/Valrubicin, Metastron/strontium derivative, Temodal/Temozolomide, Evacet/liposomal doxorubicin, Yewtaxan/Paclitaxel, Taxol/Paclitaxel, Xeloda/Capecitabine, Furtulon/Doxifluridine, Cyclopax/oral paclitaxel, Oral Taxoid, SPU-077/Cisplatin, HMR 1275/Flavopiridol, CP-358 (774)/EGFR, CP-609 (754)/RAS oncogene inhibitor, BMS-182751/oral platinum, UFT(Tegafur/Uracil), Ergamisol/Levamisole, Eniluracil/776C85/5FU enhancer, Campto/Levamisole, Camptosar/Irinotecan, Tumodex/Raltrexed, Leustatin/Cladribine, Paxex/Paclitaxel, Doxil/liposomal doxorubicin, Caelyx/liposomal doxorubicin, Fludara/Fludarabine, Pharmarubicin/Epirubicin, DepoCyt, ZD1839, LU 79553/Bis-Naphthalimide, LU 103793/Dolastain, Caetyx/liposomal doxorubicin, Gemzar/Gemcitabine, ZD 0473/Anormed, YM 116, Iodine seeds, CDK4 and CDK2 inhibitors, PARP inhibitors, D4809/Dexifosamide, Ifes/Mesnex/Ifosamide, Vumon/Teniposide, Paraplatin/Carboplatin, Plantinol/cisplatin, Vepeside/Etoposide, ZD 9331, Taxotere/Docetaxel, prodrug of guanine arabinoside, Taxane Analog, nitrosoureas, alkylating agents such as melphalan and cyclophosphamide, Aminoglutethimide, Asparaginase, Busulfan, Carboplatin, Chlorambucil, Cytarabine HCl, Dactinomycin, Daunorubicin HCl, Estramustine phosphate sodium, Etoposide (VP16-213), Floxuridine, Fluorouracil (5-FU), Flutamide, Hydroxyurea (hydroxycarbamide), Ifosfamide, Interferon Alfa-2a, Alfa-2b, Leuprolide acetate (LHRH-releasing factor analogue), Lomustine (CCNU), Mechlorethamine HCl (nitrogen mustard), Mercaptopurine, Mesna, Mitotane (o,p'-DDD), Mitoxantrone HCl, Octreotide, Plicamycin, Procarbazine HCl, Streptozocin, Tamoxifen citrate, Thioguanine, Thiotepa, Vinblastine sulfate, Amsacrine (m-AMSA), Azacitidine, Erthropoietin, Hexamethylmelamine (HMM), Interleukin 2, Mitoguazone (methyl-GAG; methyl glyoxal bis-guanylhydrazone; MGBG), Pentostatin (2'deoxycoformycin), Semustine (methyl-CCNU), Teniposide (VM-26) and Vindesine sulfate, but it is not so limited.

The immunotherapeutic agent may be selected from the group consisting of 3622W94, 4B5, ANA Ab, anti-FLK-2, anti-VEGF, ATRAGEN, AVASTIN (bevacizumab; Genentech), BABS, BEC2, BEXXAR (tositumomab; GlaxoSmithKline), C225, CAMPATH (alemtuzumab; Genzyme Corp.), CEACIDE, CMA 676, EMD-72000, ERBITUX (cetuximab; ImClone Systems, Inc.), Gliomab-H, GNI-250, HERCEPTIN (trastuzumab; Genentech),

IDE-C-Y2B8, ImmuRAIT-CEA, ior c5, ior egf.r3, ior t6, LDP-03, LymphoCide, MDX-11, MDX-22, MDX-210, MDX-220, MDX-260, MDX-447, MELIMMUNE-1, MELIMMUNE-2, Monopharm-C, NovoMAb-G2, Oncolym, OV103, Ovarex, Panorex, Pretarget, Quadramet, Ributaxin, RITUXAN (rituximab; Genentech), SMART 1D10 Ab, SMART ABL 364 Ab,, SMART M195, TNT, and ZENAPAX (daclizumab; Roche), but it is not so limited.

The cancer vaccine may be selected from the group consisting of EGF, Anti-idiotypic cancer vaccines, Gp75 antigen, GMK melanoma vaccine, MGV ganglioside conjugate vaccine, Her2/neu, Ovarex, M-Vax, O-Vax, L-Vax, STn-KHL theratope, BLP25 (MUC-1), liposomal idiotypic vaccine, Melacine, peptide antigen vaccines, toxin/antigen vaccines, MVA-based vaccine, PACIS, BCG vaccine, TA-HPV, TA-CIN, DISC-virus and ImmuCyst/TheraCys, but it is not so limited.

Anti-allergy medicaments

The TLR7/8 ligand and adaptor oligonucleotide of the invention can be used in conjunction with anti-allergy medicaments.

Conventional methods for treating or preventing allergy have involved the use of allergy medicaments or desensitization therapies. Some evolving therapies for treating or preventing allergy include the use of neutralizing anti-IgE antibodies. Anti-histamines and other drugs which block the effects of chemical mediators of the allergic reaction help to regulate the severity of the allergic symptoms but do not prevent the allergic reaction and have no effect on subsequent allergic responses. Desensitization therapies are performed by giving small doses of an allergen, usually by injection under the skin, in order to induce an IgG-type response against the allergen. The presence of IgG antibody helps to neutralize the production of mediators resulting from the induction of IgE antibodies, it is believed. Initially, the subject is treated with a very low dose of the allergen to avoid inducing a severe reaction and the dose is slowly increased. This type of therapy is dangerous because the subject is actually administered the compounds which cause the allergic response and severe allergic reactions can result.

Allergy medicaments include, but are not limited to, anti-histamines, corticosteroids, and prostaglandin inducers. Anti-histamines are compounds which counteract histamine released by mast cells or basophils. These compounds are well known in the art and commonly used for the treatment of allergy. Anti-histamines include, but are not limited to, acrivastine, astemizole, azatadine, azelastine, betastatine, brompheniramine, buclizine,

cetirizine, cetirizine analogues, chlorpheniramine, clemastine, CS 560, cyproheptadine, desloratadine, dexchlorpheniramine, ebastine, epinastine, fexofenadine, HSR 609, hydroxyzine, levocabastine, loratadine, methscopolamine, mizolastine, norastemizole, phenindamine, promethazine, pyrilamine, terfenadine, and tralast.

Corticosteroids include, but are not limited to, methylprednisolone, prednisolone, prednisone, beclomethasone, budesonide, dexamethasone, flunisolide, fluticasone propionate, and triamcinolone. Although dexamethasone is a corticosteroid having anti-inflammatory action, it is not regularly used for the treatment of allergy or asthma in an inhaled form because it is highly absorbed and it has long-term suppressive side effects at an effective dose. Dexamethasone, however, can be used according to the invention for treating allergy or asthma because when administered in combination with a composition of the invention it can be administered at a low dose to reduce the side effects. Some of the side effects associated with corticosteroid use include cough, dysphonia, oral thrush (candidiasis), and in higher doses, systemic effects, such as adrenal suppression, glucose intolerance, osteoporosis, aseptic necrosis of bone, cataract formation, growth suppression, hypertension, muscle weakness, skin thinning, and easy bruising. Barnes & Peterson (1993) *Am Rev Respir Dis* 148:S1-S26; and Kamada AK et al. (1996) *Am J Respir Crit Care Med* 153:1739-48.

Anti-asthma medicaments

The TLR 7/8 ligand and adaptor oligonucleotide combination of the invention can be used in conjunction with anti-asthma medicaments.

Medications for the treatment of asthma (i.e., anti-asthma medicaments) are generally separated into two categories, quick-relief medications and long-term control medications. Asthma patients take the long-term control medications on a daily basis to achieve and maintain control of persistent asthma. Long-term control medications include anti-inflammatory agents such as corticosteroids, chromolyn sodium and nedocromil; long-acting bronchodilators, such as long-acting β_2 -agonists and methylxanthines; and leukotriene modifiers. The quick-relief medications include short-acting β_2 agonists, anti-cholinergics, and systemic corticosteroids. There are many side effects associated with each of these drugs and none of the drugs alone or in combination is capable of preventing or completely treating asthma.

Asthma medicaments include, but are not limited, PDE-4 inhibitors, bronchodilator/beta-2 agonists, K⁺ channel openers, VLA-4 antagonists, neurokin

antagonists, thromboxane A2 (TXA2) synthesis inhibitors, xanthines, arachidonic acid antagonists, 5 lipoxygenase inhibitors, TXA2 receptor antagonists, TXA2 antagonists, inhibitor of 5-lipox activation proteins, and protease inhibitors.

Bronchodilator/ β_2 agonists are a class of compounds which cause bronchodilation or smooth muscle relaxation. Bronchodilator/ β_2 agonists include, but are not limited to, salmeterol, salbutamol, albuterol, terbutaline, D2522/formoterol, fenoterol, bitolterol, pirbuterol methylxanthines and orciprenaline. Long-acting β_2 agonists and bronchodilators are compounds which are used for long-term prevention of symptoms in addition to the anti-inflammatory therapies. Long-acting β_2 agonists include, but are not limited to, salmeterol and albuterol. These compounds are usually used in combination with corticosteroids and generally are not used without any inflammatory therapy. They have been associated with side effects such as tachycardia, skeletal muscle tremor, hypokalemia, and prolongation of QTc interval in overdose.

Methylxanthines, including for instance theophylline, have been used for long-term control and prevention of symptoms. These compounds cause bronchodilation resulting from phosphodiesterase inhibition and likely adenosine antagonism. Dose-related acute toxicities are a particular problem with these types of compounds. As a result, routine serum concentration must be monitored in order to account for the toxicity and narrow therapeutic range arising from individual differences in metabolic clearance. Side effects include tachycardia, tachyarrhythmias, nausea and vomiting, central nervous system stimulation, headache, seizures, hematemesis, hyperglycemia and hypokalemia. Short-acting β_2 agonists include, but are not limited to, albuterol, bitolterol, pirbuterol, and terbutaline. Some of the adverse effects associated with the administration of short-acting β_2 agonists include tachycardia, skeletal muscle tremor, hypokalemia, increased lactic acid, headache, and hyperglycemia.

Chromolyn sodium and nedocromil are used as long-term control medications for preventing primarily asthma symptoms arising from exercise or allergic symptoms arising from allergens. These compounds are believed to block early and late reactions to allergens by interfering with chloride channel function. They also stabilize mast cell membranes and inhibit activation and release of mediators from inosineophils and epithelial cells. A four to six week period of administration is generally required to achieve a maximum benefit.

Anticholinergics are generally used for the relief of acute bronchospasm. These compounds are believed to function by competitive inhibition of muscarinic cholinergic

receptors. Anticholinergics include, but are not limited to, ipratropium bromide. These compounds reverse only cholinergically-mediated bronchospasm and do not modify any reaction to antigen. Side effects include drying of the mouth and respiratory secretions, increased wheezing in some individuals, and blurred vision if sprayed in the eyes.

The compositions of the invention may also be useful for treating airway remodeling. Airway remodeling results from smooth muscle cell proliferation and/or submucosal thickening in the airways, and ultimately causes narrowing of the airways leading to restricted airflow. The compositions of the invention may prevent further remodeling and possibly even reduce tissue build-up resulting from the remodeling process.

Adjuvants

The TLR7/8 ligands and adaptor oligonucleotides of the invention may be used in combination with other agents, such as adjuvants. An adjuvant, as used herein, refers to a substance other than an antigen, a TLR7/8 ligand and an adaptor oligonucleotide that enhances immune cell activation in response to an antigen, e.g., a humoral and/or cellular immune response. Adjuvants promote the accumulation and/or activation of accessory cells to enhance antigen-specific immune responses. Adjuvants are used to enhance the efficacy of vaccines, i.e., antigen-containing compositions used to induce protective immunity against the antigen.

Adjuvants in general include adjuvants that create a depot effect, immune-stimulating adjuvants, and adjuvants that create a depot effect and stimulate the immune system. An adjuvant that creates a depot effect, as used herein, is an adjuvant that causes the antigen to be slowly released in the body, thus prolonging the exposure of immune cells to the antigen. This class of adjuvants includes but is not limited to alum (e.g., aluminum hydroxide, aluminum phosphate); emulsion-based formulations including mineral oil, non-mineral oil, water-in-oil or oil-in-water-in oil emulsion, oil-in-water emulsions such as Seppic ISA series of Montanide adjuvants (e.g., Montanide ISA 720; AirLiquide, Paris, France); MF-59 (a squalene-in-water emulsion stabilized with Span 85 and Tween 80; Chiron Corporation, Emeryville, Calif.); and PROVAX (an oil-in-water emulsion containing a stabilizing detergent and a micelle-forming agent; IDEC Pharmaceuticals Corporation, San Diego, Calif.).

An immune-stimulating adjuvant is an adjuvant that causes activation of a cell of the immune system. It may, for instance, cause an immune cell to produce and secrete cytokines.

This class of adjuvants includes but is not limited to saponins purified from the bark of the *Q. saponaria* tree, such as QS21 (a glycolipid that elutes in the 21st peak with HPLC fractionation; Aquila Biopharmaceuticals, Inc., Worcester, Mass.); poly[di(carboxylatophenoxy)phosphazene (PCPP polymer; Virus Research Institute, USA); derivatives of lipopolysaccharides such as monophosphoryl lipid A (MPL; Ribi ImmunoChem Research, Inc., Hamilton, Mont.), muramyl dipeptide (MDP; Ribi) and threonyl-muramyl dipeptide (t-MDP; Ribi); OM-174 (a glucosamine disaccharide related to lipid A; OM Pharma SA, Meyrin, Switzerland); and Leishmania elongation factor (a purified Leishmania protein; Corixa Corporation, Seattle, Wash.). This class of adjuvants also includes CpG DNA.

Adjuvants that create a depot effect and stimulate the immune system are those compounds which have both of the above-identified functions. This class of adjuvants includes but is not limited to ISCOMS (immunostimulating complexes which contain mixed saponins, lipids and form virus-sized particles with pores that can hold antigen; CSL, Melbourne, Australia); SB-AS2 (SmithKline Beecham adjuvant system #2 which is an oil-in-water emulsion containing MPL and QS21; SmithKline Beecham Biologicals [SBB], Rixensart, Belgium); SB-AS4 (SmithKline Beecham adjuvant system #4 which contains alum and MPL; SBB, Belgium); non-ionic block copolymers that form micelles such as CRL 1005 (these contain a linear chain of hydrophobic polyoxypropylene flanked by chains of polyoxyethylene; Vaxcel, Inc., Norcross, Ga.); and Syntex Adjuvant Formulation (SAF, an oil-in-water emulsion containing Tween 80 and a nonionic block copolymer; Syntex Chemicals, Inc., Boulder, Colo.).

The adjuvant may also be a lipopeptide such as Pam3Cys, a cationic polysaccharide such as chitosan, or a cationic peptide such as protamine.

Cytokines

The TLR7/8 ligand and adaptor oligonucleotide combination of the invention may also be used with cytokines. Cytokines are soluble proteins and glycoproteins produced by many types of cells that mediate inflammatory and immune reactions. Cytokines mediate communication between cells of the immune system, acting locally as well as systemically to recruit cells and to regulate their function and proliferation. Categories of cytokines include mediators and regulators of innate immunity, mediators and regulators of adaptive immunity, and stimulators of hematopoiesis. Included among cytokines are interleukins (e.g., IL-1, IL-2,

IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, and interleukins 19-32 (IL-19 - IL-32), among others), chemokines (e.g., IP-10, RANTES, MIP-1 α , MIP-1 β , MIP-3 α , MCP-1, MCP-2, MCP-3, MCP-4, eotaxin, I-TAC, and BCA-1, among others), as well as other cytokines including type 1 interferons (e.g., IFN- α and IFN- β), type 2 interferon (e.g., IFN- γ), tumor necrosis factor-alpha (TNF- α), transforming growth factor-beta (TGF- β), and various colony stimulating factors (CSFs), including GM-CSF, G-CSF, and M-CSF.

Antigens

The TLR7/8 ligand and adaptor oligonucleotide combination of the invention can be used with an antigen, optionally in a vaccine formulation. An “antigen” as used herein refers to any molecule capable of being recognized by a T-cell antigen receptor or B-cell antigen receptor. The term broadly includes any type of molecule which is recognized by a host immune system as being foreign. Antigens generally include but are not limited to cells, cell extracts, proteins, polypeptides, peptides, polysaccharides, polysaccharide conjugates, peptide and non-peptide mimics of polysaccharides and other molecules, small molecules, lipids, lipoproteins, glycolipids, polysaccharides, carbohydrates, viruses and viral extracts, and multicellular organisms such as parasites, and allergens. With respect to antigens that are proteins, polypeptides, or peptides, such antigens can include nucleic acid molecules encoding such antigens. Antigens more specifically include, but are not limited to, cancer antigens, which include cancer cells and molecules expressed in or on cancer cells; microbial antigens, which include microbes and molecules expressed in or on microbes; and allergens. Accordingly, the invention in certain embodiments provides vaccines for cancers, infectious agents, and allergens.

In various embodiments the antigen is a microbial antigen, a cancer antigen, or an allergen. A “microbial antigen” as used herein is an antigen of a microorganism and includes but is not limited to viruses, bacteria, parasites, and fungi. Such antigens include the intact microorganism as well as natural isolates and fragments or derivatives thereof and also synthetic compounds which are identical to or similar to natural microorganism antigens and induce an immune response specific for that microorganism. A compound is similar to a natural microorganism antigen if it induces an immune response (humoral and/or cellular) to a natural microorganism antigen. Such antigens are used routinely in the art and are well

known to those of ordinary skill in the art. The invention intends to embrace antigens derived from any of the infectious agents described herein.

As used herein, the terms "cancer antigen" and "tumor antigen" are used interchangeably to refer to a compound, such as a peptide, protein, lipoprotein or glycoprotein, which is associated with a tumor or cancer cell and which is capable of provoking an immune response when expressed on the surface of an antigen-presenting cell in the context of a major histocompatibility complex (MHC) molecule. Cancer antigens which are differentially expressed by cancer cells and can thereby be exploited in order to target cancer cells. Cancer antigens are antigens which can potentially stimulate apparently tumor-specific immune responses. Some of these antigens are encoded, although not necessarily expressed, by normal cells. These antigens can be characterized as those which are normally silent (i.e., not expressed) in normal cells, those that are expressed only at certain stages of differentiation, and those that are temporally expressed such as embryonic and fetal antigens. Other cancer antigens are encoded by mutant cellular genes, such as oncogenes (e.g., activated *ras* oncogene), suppressor genes (e.g., mutant p53), fusion proteins resulting from internal deletions or chromosomal translocations. Still other cancer antigens can be encoded by viral genes such as those carried on RNA and DNA tumor viruses.

Cancer antigens can be prepared from cancer cells either by preparing crude extracts of cancer cells, for example, as described in Cohen PA et al. (1994) *Cancer Res* 54:1055-8, by partially purifying the antigens, by recombinant technology, or by de novo synthesis of known antigens. Cancer antigens include but are not limited to antigens that are recombinantly expressed, an immunogenic portion of, or a whole tumor or cancer or cell thereof. Such antigens can be isolated or prepared recombinantly or by any other means known in the art.

Examples of tumor antigens include MAGE, MART-1/Melan-A, gp100, dipeptidyl peptidase IV (DPPIV), adenosine deaminase-binding protein (ADAbp), cyclophilin b, colorectal associated antigen (CRC)--C017-1A/GA733, carcinoembryonic antigen (CEA) and its immunogenic epitopes CAP-1 and CAP-2, etv6, aml1, prostate specific antigen (PSA) and its immunogenic epitopes PSA-1, PSA-2, and PSA-3, prostate-specific membrane antigen (PSMA), T-cell receptor/CD3-zeta chain, MAGE-family of tumor antigens (e.g., MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (MAGE-B4), MAGE-C1, MAGE-C2, MAGE-C3, MAGE-C4, MAGE-C5), GAGE-family of tumor antigens (e.g., GAGE-1, GAGE-2, GAGE-3, GAGE-4,

GAGE-5, GAGE-6, GAGE-7, GAGE-8, GAGE-9), BAGE, RAGE, LAGE-1, NAG, GnT-V, MUM-1, CDK4, tyrosinase, p53, MUC family, HER2/neu, p21ras, RCAS1, α -fetoprotein, E-cadherin, α -catenin, β -catenin and γ -catenin, p120ctn, gp100^{Pmel117}, PRAME, NY-ESO-1, cdc27, adenomatous polyposis coli protein (APC), fodrin, Connexin 37, Ig-idiotype, p15, gp75, GM2 and GD2 gangliosides, viral products such as human papillomavirus proteins, Smad family of tumor antigens, lmp-1, P1A, EBV-encoded nuclear antigen (EBNA)-1, brain glycogen phosphorylase, SSX-1, SSX-2 (HOM-MEL-40), SSX-1, SSX-4, SSX-5, SCP-1 and CT-7, and c-erbB-2. This list is not meant to be limiting.

An “allergen” as used herein is a molecule capable of provoking an immune response characterized by production of IgE. An allergen is also a substance that can induce an allergic or asthmatic response in a susceptible subject. Thus, in the context of this invention, the term allergen means a specific type of antigen which can trigger an allergic response which is mediated by IgE antibody.

The list of allergens is enormous and can include pollens, insect venoms, animal dander dust, fungal spores and drugs (e.g., penicillin). Examples of natural animal and plant allergens include proteins specific to the following genuses: Canis (*Canis familiaris*); Dermatophagoides (e.g., *Dermatophagoides farinae*); Felis (*Felis domesticus*); Ambrosia (*Ambrosia artemisiifolia*); Lolium (e.g., *Lolium perenne* and *Lolium multiflorum*); Cryptomeria (*Cryptomeria japonica*); Alternaria (*Alternaria alternata*); Alder; Alnus (*Alnus glutinosa*); Betula (*Betula verrucosa*); Quercus (*Quercus alba*); Olea (*Olea europa*); Artemisia (*Artemisia vulgaris*); Plantago (e.g., *Plantago lanceolata*); Parietaria (e.g., *Parietaria officinalis* and *Parietaria judaica*); Blattella (e.g., *Blattella germanica*); Apis (e.g., *Apis mellifera*); Cupressus (e.g., *Cupressus sempervirens*, *Cupressus arizonica* and *Cupressus macrocarpa*); Juniperus (e.g., *Juniperus sabina*, *Juniperus virginiana*, *Juniperus communis*, and *Juniperus ashei*); Thuya (e.g., *Thuya orientalis*); Chamaecyparis (e.g., *Chamaecyparis obtusa*); Periplaneta (e.g., *Periplaneta americana*); Agropyron (e.g., *Agropyron repens*); Secale (e.g., *Secale cereale*); Triticum (e.g., *Triticum aestivum*); Dactylis (e.g., *Dactylis glomerata*); Festuca (e.g., *Festuca elatior*); Poa (e.g., *Poa pratensis* and *Poa compressa*); Avena (e.g., *Avena sativa*); Holcus (e.g., *Holcus lanatus*); Anthoxanthum (e.g., *Anthoxanthum odoratum*); Arrhenatherum (e.g., *Arrhenatherum elatius*); Agrostis (e.g., *Agrostis alba*); Phleum (e.g., *Phleum pratense*); Phalaris (e.g., *Phalaris arundinacea*); Paspalum (e.g., *Paspalum notatum*); Sorghum (e.g., *Sorghum halepensis*); and Bromus (e.g., *Bromus inermis*).

Antibodies and ADCC

The TLR7/8 ligand and adaptor oligonucleotide combination of the invention also increases natural killer cell lytic activity and antibody-dependent cellular cytotoxicity (ADCC). ADCC can be performed using the combination with an antibody specific for a cellular target, such as a cancer cell, leading the subject's immune system to kill the tumor cell. The antibodies useful in the ADCC procedure include antibodies which interact with a cell in the body. Many such antibodies specific for cellular targets have been described in the art and many are commercially available. In one embodiment the antibody is an IgG antibody.

Therapeutic antibodies useful in the invention may be specific for microbial antigens (e.g., bacterial, viral, parasitic or fungal antigens), cancer or tumor-associated antigens and self antigens. Preferred antibodies are those that recognize and bind to antigens present on or in a cell. Examples of suitable antibodies include but are not limited to Rituxan™ (rituximab, anti-CD20 antibody), Herceptin (trastuzumab), Quadramet, Panorex, IDEC-Y2B8, BEC2, C225, Oncolym, SMART M195, ATRAGEN, Ovarex, Bexxar, LDP-03, ior t6, MDX-210, MDX-11, MDX-22, OV103, 3622W94, anti-VEGF, Zenapax, MDX-220, MDX-447, MELIMMUNE-2, MELIMMUNE-1, CEACIDE, Pretarget, NovoMAb-G2, TNT, Gliomab-H, GNI-250, EMD-72000, LymphoCide, CMA 676, Monopharm-C, 4B5, ior egf.r3, ior c5, BABS, anti-FLK-2, MDX-260, ANA Ab, SMART 1D10 Ab, SMART ABL 364 Ab, CC49 (mAb B72.3), ImmuRAIT-CEA, anti-IL-4 antibody, an anti-IL-5 antibody, an anti-IL-9 antibody, an anti-Ig antibody, an anti-IgE antibody, serum-derived hepatitis B antibodies, recombinant hepatitis B antibodies, and the like.

Other antibodies similarly useful for the invention include alemtuzumab (B cell chronic lymphocytic leukemia), gentuzumab ozogamicin (CD33+ acute myeloid leukemia), hP67.6 (CD33+ acute myeloid leukemia), infliximab (inflammatory bowel disease and rheumatoid arthritis), etanercept (rheumatoid arthritis), tositumomab, MDX-210, oregovomab, anti-EGF receptor mAb, MDX-447, anti-tissue factor protein (TF), (Sunol); ior-c5, c5, edrecolomab, ibritumomab tiuxetan, anti-idiotypic mAb mimic of ganglioside GD3 epitope, anti-HLA-Dr10 mAb, anti-CD33 humanized mAb, anti-CD52 humAb, anti-CD1 mAb (ior t6), MDX-22, celogovab, anti-17-1A mAb, bevacizumab, daclizumab, anti-TAG-72 (MDX-220), anti-idiotypic mAb mimic of high molecular weight proteoglycan (I-Mel-1), anti-idiotypic mAb mimic of high molecular weight proteoglycan (I-Mel-2), anti-CEA Ab,

hmAbH11, anti-DNA or DNA-associated proteins (histones) mAb, Gliomab-H mAb, GNI-250 mAb, anti-CD22, CMA 676), anti-idiotypic human mAb to GD2 ganglioside, ior egf/r3, anti-ior c2 glycoprotein mAb, ior c5, anti-FLK-2/FLT-3 mAb, anti-GD-2 bispecific mAb, antinuclear autoantibodies, anti-HLA-DR Ab, anti-CEA mAb, palivizumab, bevacizumab, alemtuzumab, BLyS-mAb, anti-VEGF2, anti-Trail receptor; B3 mAb, mAb BR96, breast cancer; and Abx-Cbl mAb.

Immunoglobulin Therapy

The agents of the invention can also be used with normal and hyper-immune globulin therapy. Normal immune globulin therapy utilizes an antibody product which is prepared from the serum of normal blood donors and pooled. This pooled product contains low titers of antibody to a wide range of antigens such as those of infectious pathogens (e.g., bacteria, viruses such as hepatitis A, parvovirus, enterovirus, fungi and parasites). Hyper-immune globulin therapy utilizes antibodies which are prepared from the serum of individuals who have high titers of an antibody to a particular antigen. Examples of hyper-immune globulins include zoster immune globulin (useful for the prevention of varicella in immunocompromised children and neonates), human rabies immunoglobulin (useful in the post-exposure prophylaxis of a subject bitten by a rabid animal), hepatitis B immune globulin (useful in the prevention of hepatitis B virus, especially in a subject exposed to the virus), and RSV immune globulin (useful in the treatment of respiratory syncytial virus infections).

Dosing and Administration

The ligand and oligonucleotide can be formulated in separate compositions that are used together to achieve a desired effect. For example, an adaptor oligonucleotide and a TLR7/8 ligand can be mixed together and administered to a subject or placed in contact with a cell substantially simultaneously as a combination. As another example, an adaptor oligonucleotide and a TLR7/8 ligand can be administered to a subject or placed in contact with a cell at different times. As yet another example, an adaptor oligonucleotide and a TLR7/8 ligand can be administered to a subject at different sites.

As mentioned above, the term "effective amount" refers generally to the amount necessary or sufficient to realize a desired biologic effect. Combined with the teachings provided herein, by choosing among the various active compounds and weighing factors such as potency, relative bioavailability, patient body weight, severity of adverse side-effects and

preferred mode of administration, an effective prophylactic or therapeutic treatment regimen can be planned which does not cause substantial toxicity and yet is entirely effective to treat the particular subject. The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular oligonucleotide being administered, the size of the subject, or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular adaptor oligonucleotide and TLR7/8 ligand and/or antigen and/or other therapeutic agent without necessitating undue experimentation.

Subject doses of the compounds described herein for systemic or local delivery typically range from about 10 ng to 10 mg per administration, which depending on the application could be given daily, weekly, or monthly and any other amount of time therebetween or as otherwise required. More typically systemic or local doses range from about 1 µg to 1 mg per administration, and most typically from about 10 µg to 100 µg, with 2 - 4 administrations being spaced days or weeks apart. Higher doses may be required for parenteral administration. In some embodiments, however, parenteral doses for these purposes may be used in a range of 5 to 10,000 times higher than the typical doses described above.

For any compound described herein the therapeutically effective amount can be initially determined from animal models. The applied dose can be adjusted based on the relative bioavailability and potency of the administered compound. Adjusting the dose to achieve maximal efficacy based on the methods described above and other methods as are well-known in the art is well within the capabilities of the ordinarily skilled artisan.

Route of Administration

For clinical use the TLR7/8 ligand and adaptor oligonucleotide combination of the invention can be administered alone or formulated as a delivery complex via any suitable route of administration that is effective to achieve the desired therapeutic result. Routes of administration include enteral and parenteral routes of administration. Examples of enteral routes of administration include oral, gastric, intestinal, and rectal. Nonlimiting examples of parenteral routes of administration include intravenous, intramuscular, subcutaneous, intraperitoneal, intrathecal, local injection, topical, nasal, mucosal, and pulmonary.

Delivery Vehicles

The adaptor oligonucleotide and a TLR7/8 ligand and/or the antigen and/or other therapeutics may be administered alone (e.g., in saline or buffer) or using any delivery vehicle known in the art.

For example, the TLR7/8 ligand and adaptor oligonucleotide combination of the invention may be directly administered to the subject or may be administered in conjunction with a nucleic acid delivery complex. A nucleic acid delivery complex shall mean a nucleic acid molecule associated with (e.g., ionically or covalently bound to; or encapsulated within) a targeting means (e.g., a molecule that results in higher affinity binding to target cell). Examples of nucleic acid delivery complexes include nucleic acids associated with a sterol (e.g., cholesterol), a lipid (e.g., a cationic lipid, virosome or liposome), or a target cell specific binding agent (e.g., a ligand recognized by target cell specific receptor). Preferred complexes may be sufficiently stable *in vivo* to prevent significant uncoupling prior to internalization by the target cell. However, the complex can be cleavable under appropriate conditions within the cell so that the oligonucleotide is released in a functional form.

Other delivery vehicles that have been described and can be used according to the invention include cochleates (Gould-Fogerite et al., 1994, 1996); emulsomes (Vancott et al., 1998, Lowell et al., 1997); ISCOMs (Mowat et al., 1993, Carlsson et al., 1991, Hu et., 1998, Morein et al., 1999); liposomes (Childers et al., 1999, Michalek et al., 1989, 1992, de Haan 1995a, 1995b); live bacterial vectors (e.g., *Salmonella*, *Escherichia coli*, bacillus Calmette-Guérin, *Shigella*, *Lactobacillus*) (Hone et al., 1996, Pouwels et al., 1998, Chatfield et al., 1993, Stover et al., 1991, Nugent et al., 1998); live viral vectors (e.g., Vaccinia, adenovirus, Herpes simplex) (Gallichan et al., 1993, 1995, Moss et al., 1996, Nugent et al., 1998, Flexner et al., 1988, Morrow et al., 1999); microspheres (Gupta et al., 1998, Jones et al., 1996, Maloy et al., 1994, Moore et al., 1995, O'Hagan et al., 1994, Eldridge et al., 1989); nucleic acid vaccines (Fynan et al., 1993, Kuklin et al., 1997, Sasaki et al., 1998, Okada et al., 1997, Ishii et al., 1997); polymers (e.g., carboxymethylcellulose, chitosan) (Hamajima et al., 1998, Jabbal-Gill et al., 1998); polymer rings (Wyatt et al., 1998); proteosomes (Vancott et al., 1998, Lowell et al., 1988, 1996, 1997); sodium fluoride (Hashi et al., 1998); transgenic plants (Tacket et al., 1998, Mason et al., 1998, Haq et al., 1995); Virosomes (Gluck et al., 1992, Mengardi et al., 1995, Cryz et al., 1998); virus-like particles (Jiang et al., 1999, Leibl et al., 1998). Other delivery vehicles are known in the art.

Pharmaceutical Compositions

The TLR7/8 ligand and adaptor oligonucleotide combinations of the invention can be formulated as pharmaceutical compositions additionally comprising a pharmaceutically acceptable carrier. A pharmaceutically-acceptable carrier means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration to a human or other vertebrate animal. A carrier is an organic or inorganic ingredient, natural or synthetic, with which the active ingredient(s) are combined to facilitate the desired effect. The components of a pharmaceutical composition are co-mingled in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficiency.

For oral administration, the compounds (i.e., adaptor oligonucleotide and a TLR7/8 ligand, antigens and/or other therapeutic agents) can be formulated readily by combining the active compound(s) with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject to be treated. Pharmaceutical preparations for oral use can be obtained as solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Optionally the oral formulations may also be formulated in saline or buffers for neutralizing internal acid conditions or may be administered without any carriers.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol

or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. Microspheres formulated for oral administration may also be used. Such microspheres have been well defined in the art. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

The compounds may be administered by inhalation to pulmonary tract, especially the bronchi and more particularly into the alveoli of the deep lung, using standard inhalation devices. The compounds may be delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide, or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. An inhalation apparatus may be used to deliver the compounds to a subject. An inhalation apparatus, as used herein, is any device for administering an aerosol, such as dry powdered form of the compounds. This type of equipment is well known in the art and has been described in detail, such as that description found in Remington: The Science and Practice of Pharmacy, 19th Edition, 1995, Mac Publishing Company, Easton, Pennsylvania, pages 1676-1692. Many U.S. patents also describe inhalation devices, such as U.S. Pat. No. 6,116,237.

“Powder” as used herein refers to a composition that consists of finely dispersed solid particles. Preferably the compounds are relatively free flowing and capable of being dispersed in an inhalation device and subsequently inhaled by a subject so that the compounds reach the lungs to permit penetration into the alveoli. A “dry powder” refers to a powder composition that has a moisture content such that the particles are readily dispersible in an inhalation device to form an aerosol. The moisture content is generally below about 10% by weight (% w) water, and in some embodiments is below about 5% w and preferably less than about 3% w. The powder may be formulated with polymers or optionally may be formulated with other materials such as liposomes, albumin and/or other carriers.

Aerosol dosage and delivery systems may be selected for a particular therapeutic application by one of skill in the art, such as described, for example in Gonda, I. “Aerosols

for delivery of therapeutic and diagnostic agents to the respiratory tract," in Critical Reviews in Therapeutic Drug Carrier Systems, 6:273-313 (1990), and in Moren, "Aerosol dosage forms and formulations," in Aerosols in Medicine. Principles, Diagnosis and Therapy, Moren, et al., Eds., Elsevier, Amsterdam, 1985.

The compounds, when it is desirable to deliver them systemically, may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active compounds may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal or vaginal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The pharmaceutical compositions also may include suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Suitable liquid or solid pharmaceutical preparation forms are, for example, aqueous or saline solutions for inhalation, microencapsulated, encochleated, coated onto microscopic gold particles, contained in liposomes, nebulized, aerosols, pellets for implantation into the skin, or dried onto a sharp object to be scratched into the skin. The pharmaceutical compositions also include granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, drops or preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners or solubilizers are customarily used as described above. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems. For a brief review of methods for drug delivery, see Langer R (1990) *Science* 249:1527-33, which is incorporated herein by reference.

The agents of the invention and optionally other therapeutics and/or antigens may be administered *per se* (neat) or in the form of a pharmaceutically acceptable salt. When used in medicine the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof. Such salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene sulphonic, tartaric, citric, methane sulphonic, formic, malonic, succinic, naphthalene-2-sulphonic, and benzene sulphonic. Also, such salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group.

Suitable buffering agents include: acetic acid and a salt (1-2% w/v); citric acid and a salt (1-3% w/v); boric acid and a salt (0.5-2.5% w/v); and phosphoric acid and a salt (0.8-2% w/v). Suitable preservatives include benzalkonium chloride (0.003-0.03% w/v); chlorobutanol (0.3-0.9% w/v); parabens (0.01-0.25% w/v) and thimerosal (0.004-0.02% w/v).

The pharmaceutical compositions of the invention contain an effective amount of the agents of the invention and optionally antigens and/or other therapeutic agents optionally included in a pharmaceutically acceptable carrier. The term pharmaceutically acceptable carrier means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration to a human or other vertebrate animal. The term carrier denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being commingled with the compounds of

the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficiency.

Screening Methods

The invention provides in yet other aspects methods for identifying TLR7/8 ligands and/or adaptor oligonucleotides. The method steps vary depending on the agent being identified (i.e., ligand versus oligonucleotide), whether the ligand is a TLR7, a TLR8 or a TLR7/8 ligand, the type of cells used in the method, and the like.

Based on the teachings set forth herein, a TLR7 ligand may be identified by its ability to stimulate TLR7 signaling alone, to stimulate TLR8 signaling (starting from a background level) in the presence of an adaptor oligonucleotide, and/or by inhibition of TLR7 stimulation in the presence of the adaptor oligonucleotide. A TLR8 ligand may be identified by enhanced TLR8 signaling (starting from an above background level) when used in combination with an adaptor oligonucleotide. A ligand that stimulates both TLR7 and 8 (when used alone) can be identified by any combination of these activities. An adaptor oligonucleotide may be identified by its ability to inhibit TLR7 stimulation by a TLR7 ligand, induce TLR8 stimulation by a TLR7 ligand, and/or enhance TLR8 stimulation from a TLR8 ligand.

The methods may be performed in vivo or in vitro. In vitro assays are documented in the Examples. Suitable readouts include but are not limited to IL-12, TNF-alpha and/or IFN-gamma for TLR8 stimulation, and IFN-alpha for TLR7 stimulation. The assays may alternatively employ reporter constructs having reporter genes linked to transcriptional regulation elements that are responsive to TLR7 and/or TLR8 signaling (e.g., NF- κ B responsive elements). These constructs are described in the Examples. The assays may measure transcriptional up- or down-regulation, translational up- or down-regulation, protein expression and/or secretion, and the like.

As an example, a TLR8 ligand assay may involve contacting a TLR8-expressing cell (or cell population) with a test ligand in the presence and absence of an adaptor oligonucleotide. The cell preferably is one that expresses TLR8 but not TLR7 or TLR9. TLR8 signaling is measured from the cell in response to the test ligand in the presence and absence of the oligonucleotide. A test ligand having a TLR8 signaling profile that is enhanced in the presence of the oligonucleotide, as compared to in the absence of the oligonucleotide, is then identified as a TLR8 ligand. The assay may also include an analysis of TLR7 signaling using a TLR7-expressing cell that does not express TLR8 or TLR9. This

latter analysis can be used to exclude the possibility that the ligand is a TLR7 ligand that switches to TLR8 signaling in the presence of the oligonucleotide.

Results from a similar assay using known ligands is shown in FIG. 12. Both loxoribine and immunosine (TLR7 specific ligands) stimulate TLR8 signaling in the presence of ODN 6056. Putative TLR7 specific ligands would be expected to have a qualitatively similar profile. TLR8 signaling by R-848 (a TLR7 and TLR8 ligand) is enhanced in the presence of ODN 6056. Putative TLR8 ligands would be expected to have a qualitatively similar profile. Ribavirin, which is neither a TLR7 nor a TLR8 ligand, does not stimulate TLR8 signaling in the presence or absence of ODN 6056, indicating the specificity of the assay for TLR7/8 ligands.

An adaptor oligonucleotide assay may involve contacting a TLR8-expressing cell (or cell population) with a test oligonucleotide in the presence and absence of a TLR7, TLR8 or TLR7/8 ligand. If the ligand is a TLR7 ligand (when used alone), the assay may measure TLR7 signaling inhibition and/or induced TLR8 signaling when the oligonucleotide is used with the ligand. If the ligand is a TLR8 ligand (when used alone), the assay may measure enhanced TLR8 signaling when the oligonucleotide is used with the ligand as compared to the effect of the ligand alone. If the ligand stimulates both TLR7 and TLR8 (when used alone), the assay may measure one or more of the above-noted readouts. The assay results may be compared to positive control assays (e.g. assays using a known adaptor oligonucleotide), and the like. The method may also include an assay for determining if the oligonucleotide is a TLR7 and/or TLR8 ligand itself.

These methods may be used to identify ligands which when used alone are weakly stimulatory, but which when combined with oligonucleotides have new and/or enhanced signaling profiles.

The present invention is further illustrated by the following Examples, which in no way should be construed as further limiting.

Examples

MATERIALS & METHODS

Oligodeoxynucleotides and reagents

All oligonucleotides were purchased from Biospring (Frankfurt, Germany) or provided by Coley Pharmaceutical GmbH (Langenfeld, Germany), controlled for identity and

purity by Coley Pharmaceutical GmbH and had undetectable endotoxin levels (<0.1EU/ml) measured by the Limulus assay (BioWhittaker, Verviers, Belgium). Oligonucleotides were suspended in sterile, endotoxin-free Tris-EDTA (Sigma, Deisenhofen, Germany), and stored and handled under aseptic conditions to prevent both microbial and endotoxin contamination. All dilutions were carried out using endotoxin-free Tris-EDTA. Sequences of oligonucleotides are listed in Tables 1 and 2. Loxoribine (7-allyl-7,8-dihydro-8-oxo-guanosine) (Sigma) was dissolved first in 1N NaOH, diluted in RPMI-medium and pH was adjusted to 7.4 with 1N HCl (19). R-848 (1-(2-hydroxy2-methylpropyl)-2-methyl-1H-imidazo[4,5-c]quinolin-4-amine) was commercially synthesized by GLSynthesis (Worcester, MA, USA) and dissolved in 10% DMSO. 7-Deaza-guanosine (ChemGenes, Wilmington, MA, USA) was dissolved at 1M in 1N NaOH. Inosine (Sigma) was dissolved in H₂O. All dilutions were done in endotoxin-free Tris-EDTA.

TLR assays

Stably transfected HEK293 cells expressing the human TLR9, TLR8 or TLR7 were described before (16,20). Briefly, HEK293 cells were transfected by electroporation with vectors expressing the respective human TLR and a 6xNF-kappaB-luciferase reporter plasmid. Stable transfectants (3×10^4 cells/well) were incubated with loxoribine or R-848 in the absence or presence of ODN for 16h at 37°C in a humidified incubator. Each data point was done in triplicate. Cells were lysed and assayed for luciferase gene activity (using the BriteLite kit from Perkin-Elmer, Zaventem, Belgium). Stimulation indices were calculated in reference to reporter gene activity of medium without addition of oligonucleotide.

Cell purification

Peripheral blood buffy coat preparations from healthy human donors were obtained from the Blood Bank of the University of Düsseldorf (Germany) and PBMC were purified by centrifugation over Ficoll-Hypaque (Sigma). Cells were cultured in a humidified incubator at 37°C in RPMI 1640 medium supplemented with 5% (v/v) heat inactivated human AB serum (BioWhittaker) or 10% (v/v) heat inactivated FCS, 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin (all from Sigma).

Cytokine detection and flow cytometric analysis

PBMC were resuspended at a concentration of 5×10^6 cells/ml and added to 96 well round-bottomed plates (250 µl/well). PBMC were incubated with various ODN and/or loxoribine concentrations and culture supernatants (SN) were collected after the indicated time points. If not used immediately, SN were stored at -20°C until required. Amounts of cytokines in the SN were assessed using a commercially available ELISA Kit (for IL-12p40, from BD Biosciences, Heidelberg, Germany), IFN- γ and TNF- α (from Diaclone, Besançon, France) or an in-house ELISA for IFN- γ developed using commercially available antibodies (PBL, New Brunswick, NJ, USA).

For intracellular staining, PBMC were incubated at 5×10^6 cells/ml in 96 well round-bottomed plates (250 µl/well) with indicated amounts of oligonucleotides and/or loxoribine concentrations, and Brefeldin A solution (BD Biosciences) was added. PBMC were incubated for 6h. For intracellular IFN- γ staining, cells were incubated with oligonucleotides and/or loxoribine for 16h before addition of Brefeldin A solution. Cells were harvested and intracellular staining performed using Intraprep reagent according to the manufacturer protocol (Beckman-Coulter, Neuss, Germany). Cells were stained with appropriate antibodies for identification of monocytes (CD14 $^+$), B cells (CD19 $^+$), and NK cells (CD56 $^+$, CD3 $^+$). Flow cytometric data were acquired on a FACSCalibur and were analyzed using the computer program CellQuest (both from BD Biosciences). All monoclonal antibodies (mAb) for flow cytometric analysis were purchased from BD Biosciences, except CD11c from Diaclone, CD14 from Immunotech (Marseille, France) and CD123 from Miltenyi (Bergisch Gladbach, Germany). Human monocytes were isolated from whole PBMC using the CD14 cell isolation kit as described by the manufacturer (Miltenyi). To determine purity, cells were stained with mAb to CD11c and CD14 and identified by flow cytometry. In all experiments, monocytes were more than 95% pure. Purified monocytes (4×10^6 cells/ml) were incubated with increasing concentrations of ODN for 24h. PDC were enriched using the BDCA-4 pDC isolation kit as described by the manufacturer (Miltenyi). PDC purification was confirmed by staining with mAb to CD123 (from Miltenyi), HLA-DR and CD11c (from BD Biosciences). Purity was approximately 85%. Cells (5×10^5 cells/ml, 250 µl/well) were cultured for 24h with or without oligonucleotides and loxoribine. IFN- α or IL-12p40 in the SN was measured as described above.

RESULTS

Sequence-selective enhancement of TLR8-mediated signaling by co-incubation with homopolymer oligonucleotide

HEK293 cells stably expressing hTLR8 and a NF- κ B-luciferase reporter construct were incubated with R-848 (1-(2-hydroxy-2-methylpropyl)-2-methyl-1H-imidazo[4,5-c]quinolin-4-amine) in the presence or absence of oligonucleotide. Influence of co-incubation with different oligonucleotides on TLR8-mediated NF- κ B activation was then analyzed.

An oligo(dT)₁₇ homopolymer ODN with a phosphorothioate backbone, ODN 6056, was identified that markedly increased the level of NF- κ B activation stimulated by R-848 (FIG. 1B). ODN 6056 alone did not stimulate considerable NF- κ B activation (not more than 2.5 fold above background) at concentrations up to 25 μ M in TLR8-expressing cells (unpublished data). The effect appeared to be oligonucleotide-dependent as not all of the oligonucleotides tested enhanced TLR8 signaling equally well (e.g., an unrelated heteropolymer (ODN 1982) did not strongly influence NF- κ B activation by R-848).

Co-incubation of R-848 with ODN 6056 not only increased efficacy but also the potency of TLR8 activation significantly. The EC50 of R-848 was already strongly decreased in the presence of 0.1 μ M ODN 6056 (0.1 μ M ODN 6056: EC50 (R-848) = 4.9 μ M, mean from three experiments; EC50 (R-848) alone > 30 μ M). However, higher concentrations of ODN 6056 decreased the EC50 even more (1 μ M ODN 6056: EC50 (R-848) = 1.4 μ M; 5 μ M ODN: EC50 (R-848) = 0.4 μ M). This effect was specific for ODN 6056 as the decrease of EC50 was considerably less for ODN 1982 (5 μ M 1982: EC50 (R-848) = 19.0 μ M). In contrast to the stimulatory effects on TLR8, co-incubation of ODN 6056 (or ODN 1982) with the TLR9 ligand CpG ODN 2006 on hTLR9 expressing HEK293 cells did not affect CpG-mediated NF- κ B stimulation (unpublished data).

The potential sequence requirements for the observed enhancement effect was investigated and several different phosphorothioate homopolymers were tested (Table 1). The oligo(dT)₁₇ (ODN 6056) had the strongest enhancing properties, followed by an oligo(dU)₁₇ (SEQ ID NO: 11) and oligo(dA)₁₇ (SEQ ID NO: 12). Significantly less enhancement was detected for an oligo(dC)₁₇ (SEQ ID NO: 13) and a randomised oligo(dN)₁₅. The heteropolymer ODN 1982 showed only minor enhancing activity and an oligo(dG)₂₄ (SEQ ID NO: 14) did not enhance activity, but rather inhibited R-848-mediated NF- κ B stimulation. The sequence of ODN 6056 with a phosphodiester backbone did not influence TLR8-mediated stimulation. Length of the oligonucleotide also seemed to have an influence: an

oligo(dA)₂₄ (SEQ ID NO: 15) showed less synergistic effects with R-848 than the shorter oligo(dA)₁₇ (SEQ ID NO: 12).

Stimulation of TLR8-expressing cells by a TLR7-specific ligand in the presence of a homopolymer T oligonucleotide

R-848 is a ligand for hTLR7 as well as hTLR8 (16), whereas loxoribine (7-allyl-7,8-dihydro-8oxo-guanosine) was described as a TLR7-specific ligand (18). Concentrations of up to 10 mM loxoribine did not activate NF- κ B signaling in HEK293 cells transfected with either hTLR8 or hTLR9, but activated hTLR7-mediated signaling (FIG. 2A). Although being a TLR7 ligand, co-incubation of hTLR8-expressing HEK293 cells with loxoribine and oligonucleotide activated NF- κ B signaling (FIG. 2B). This concentration-dependent effect was observed exclusively in the presence of ODN 6056 but not ODN 1982 (FIG. 2B). Similar observations were made with another TLR7 ligand, 7-deaza-guanosine (18). 7-Deaza-guanosine alone was inactive on hTLR8-expressing cells at concentrations of up to 5 mM in the absence of ODN 6056 but stimulated TLR8-mediated NF- κ B activation in the presence of ODN 6056. Inosine, a compound that appeared to activate NF- κ B signaling in HEK293 cells nonspecifically (unpublished data), was also co-incubated with ODN 6056 on hTLR8-expressing HEK293 cells (FIG. 2C). NF- κ B activation by inosine in the presence of ODN 6056 was not altered compared to nonspecific activation of inosine at concentrations of up to 10 mM.

A similar effect is observed with 6-amino-9-benzyl-2-butoxy-9H-purin-8-ol, an adenosine-based compound. When used alone, this compound is a TLR7 ligand but not a TLR8 ligand (FIGs. 9A and 9B). When used in the presence of a poly(dT)₁₇ adaptor oligonucleotide, it is able to stimulate TLR8 signaling (FIG. 9B).

Sequence-independent inhibition of TLR7 signaling

These data show that small molecule TLR7 ligands induce TLR8-dependent NF- κ B signaling in the presence of certain oligonucleotides. Nevertheless, in TLR7-expressing HEK293 cells the effects were converse. Oligonucleotides actually inhibited TLR7-mediated NF- κ B activation by TLR7 specific ligands and TLR7/8 ligands as shown in FIGs. 1A, 3 and 9A. The effect appeared to be sequence-unspecific as all phosphorothioate oligonucleotides tested so far inhibited hTLR7 activation (unpublished data).

Enhancement of TLR8 ligand signaling using an RNA adaptor oligonucleotide

Similar experiments were carried out using R-848 as the TLR7/8 ligand and RNA-based adaptor oligonucleotides. FIG. 10 shows the effect on NF- κ B stimulation in hTLR8-LUC-293 cells by a mixed sequence oligonucleotide and a poly(rU)₁₈ oligonucleotide. The poly(rU)₁₈ oligonucleotide

(rU*rU*rU*rU*rU*rU*rU*rU*rU*rU*rU*rU*rU*rU*rU*rU*rU; SEQ ID NO: 5) when used alone signals through TLR8, as shown by the slight activation of NF- κ B with this oligonucleotide. (FIG. 10) The mixed sequence RNA oligonucleotide

(rG*rC*rC*rA*rC*rG*rA*rG*rC*rC*rG*rA*rA*rG*rG*rC*rA*rC*rC; SEQ ID NO: 16), when used alone, does not signal through TLR8.

In FIG. 11, hTLR8-LUC-HEK293 cells were incubated with increasing amounts of R-848 in the presence of either (a) TE and 50 μ g/ml DOTAP, or (b) 5 μ M of indicated ORN in the presence of 50 μ g/ml DOTAP. Stimulation of NF- κ B was measured 16 hours later by determining luciferase activity. Stimulation indices were calculated in reference to background in the presence of medium alone. The Figure shows that the effect of the poly(rU)₁₈ oligonucleotide on TLR8 signaling is dramatically enhanced when the oligonucleotide is combined with R-848 (FIG. 11). The mixed sequence RNA oligonucleotide, which is neither a TLR7 nor TLR8 ligand, also enhances TLR8 signaling by R-848 as shown in FIG. 11. The data show that RNA-based oligonucleotides can also function as adaptors.

Co-incubation of loxoribine with oligo(dT)₁₇ ODN changes the cytokine profile produced by human PBMC

The effect of co-incubation of loxoribine with ODN 6056 and 1982 on human immune cells was investigated. Incubation with loxoribine alone stimulated human PBMC to produce IFN- α (FIG. 4A). However, in the presence of increasing concentrations of ODN, loxoribine-induced IFN- α was reduced to background levels. Although the effect was observed with both ODN, ODN 6056 seemed to have a stronger suppressive effect.

The production of other cytokines was also studied. Loxoribine alone induced only minor amounts of IL-12p40 secretion from human PBMC (FIG. 4B). In contrast, in the presence of ODN 6056 IL-12p40 was produced in significant amounts, whereas the same concentration of ODN 1982 together with loxoribine did not induce IL-12p40 above the background with media alone. Similar results were obtained for IL-12p70 (unpublished data)

and IFN- γ (FIG. 4C). TNF- α production was also stimulated when loxoribine and ODN 6056 were co-incubated. However, TNF- α production was also observed when ODN 1982 was co-incubated with loxoribine, although considerably less than achieved by co-incubation with ODN 6056. None of the ODN nor loxoribine alone induced significant levels of TNF- α secretion (FIG. 4D).

IL-12p40 and TNF- α are produced by monocytes when co-stimulated with loxoribine and oligo(dT)₁₇ ODN

The combination of loxoribine and ODN activates TLR8-expressing HEK293 cells but suppresses TLR7-mediated signaling. It was hypothesized that human TLR8-positive immune cells are the primary source for IL-12 and TNF- α and TLR7-positive pDCs are the primary source for IFN- α . Intracellular FACS staining revealed that the majority of CD14+ cells produced TNF- α upon co-incubation of human PBMC with loxoribine and ODN 6056 (FIG. 5A). Loxoribine alone or in combination with ODN 1982 yielded only few TNF- α -positive monocytes. The percentage of IL-12-positive monocytes was less than that for TNF- α . Nevertheless, the synergistic effect of the combination of loxoribine with ODN 6056 was also clearly detectable for intracellular IL-12 (FIG. 5B). In none of these experiments could intracellular IL-12 or TNF- α be detected in CD19+ B cells upon stimulation with ODN and loxoribine (unpublished data). The cellular source of IFN- γ was also investigated. The combination of loxoribine with ODN stimulated high IL-12 levels and previous studies showed that IL-12 induces IFN- γ secretion in NK cells (21-23). Therefore, IFN- γ production was evaluated in human NK cells (FIG. 6). Indeed, IFN- γ production in NK cells could be observed when human PBMC were co-incubated with loxoribine and ODN 6056, although neither of the stimuli alone induced considerable IFN- γ production as already demonstrated by IFN- γ protein ELISA from supernatants of human PBMC.

To demonstrate direct TLR-mediated stimulation of monocytes in response to loxoribine and ODN 6056, monocytes from human PBMC were purified and incubated with loxoribine in the presence or absence of ODN 6056 or 1982. Isolated monocytes produced IL-12p40 (FIG. 7A) or TNF- α (unpublished data) only in response to the combination of both stimuli, loxoribine and ODN 6056.

Incubation of human PBMC with small immunomodulating compounds like R-848 or loxoribine induced IFN- α production by pDCs (FIG. 7B and (24,25)). Therefore, the suppressive effect of ODN 6056 on IFN- α production stimulated by loxoribine could have

been a direct result of inhibition of TLR7-mediated signaling in pDCs. To investigate this possibility, human pDCs were isolated and stimulated with loxoribine in the presence of either oligo(dT)₁₇ homopolymer or heteropolymer ODN (FIG. 7B). Enriched human pDC produced high amounts of IFN- α when incubated with loxoribine alone. In contrast, the IFN- α production was abolished when co-incubated with either ODN 6056 or ODN 1982. Again, the suppressive effect of ODN 6056 seemed to be stronger than that of ODN 1982 since lower ODN concentrations were needed to abrogate IFN- α production.

FIG. 8 shows data generated using TLR8-LUC-293 cells. A constant concentration of R-848 (50 micromolar) and increasing amounts of the specified ODN were used. Luciferase readout of R-848 alone was set to 100% for standardization. The results are shown in the left panel. FIG. 8 right panel shows the effect of increasing T content in a 1 micromolar 17mer poly C oligonucleotide in the presence of increasing amounts of R-848. The activity is enhanced even with just two thymidines and increases dramatically with 6 or more thymidines.

DISCUSSION

The present data demonstrate that the activity of hTLR8 can be modulated by the presence of certain phosphorothioate ODN. hTLR8 is most closely related to hTLR7 (26), as reflected by the ability of the small immune stimulatory compound R-848 to activate both hTLR7 and hTLR8 (16). However, hTLR7 is considerably more sensitive to stimulation with R-848 than hTLR8 as lower concentrations of R-848 are needed for TLR7 activation. Co-incubation of R848 with a phosphorothioate oligo(dT)₁₇ rendered TLR8 more sensitive to stimulation with R848 and yet inhibited TLR7 activation, despite the fact that this T homopolymer alone had no considerable effect on either of these TLRs.

To determine whether the T homopolymer would influence TLR activation by other stimuli, its effects on two TLR7 ligands, loxoribine and 7-deaza-guanosine, which do not stimulate TLR8 (18), were studied. Surprisingly, for both ligands the presence of an oligo(dT)₁₇ led to a complete switch from a TLR7- to a TLR8-dependent NF- κ B activation, with suppression of TLR7-mediated signaling by the oligo(dT)₁₇. It is possible that human TLR8 may have a weak binding affinity to loxoribine which probably cannot be detected in the biological assays available. The presence of certain oligonucleotides may enhance affinity of loxoribine to TLR8 by a yet unknown mechanism, so that loxoribine-mediated stimulation of TLR8 in stably transfected cells can be detected.

The possibility of an influence of oligonucleotides on a general signaling pathway or uptake mechanism cannot be ruled out. However, the fact that the oligo(dT)₁₇ had a different mode of action on HEK293 cells transfected with hTLR7 (inhibition of NF- κ B signaling), hTLR9 (no effect) or hTLR8 (strong enhancement of signaling) strongly argues against this possibility. The observed synergistic effect was not limited to cells expressing TLR8 ectopically. Human TLR7 is mainly expressed in pDCs and B cells. These cells do not express TLR8 whereas TLR8 is expressed in myeloid cells (27-30). Indeed, synergistic effects of loxoribine and ODN on cytokine production (IL-12 and TNF- α) were measured in human immune cells with endogenous TLR8 expression such as monocytes. In contrast, the majority of loxoribine and R-848 induced IFN- α is produced by activation of TLR7 in human pDC (our data and (4,24,25)). Activation of IFN- α production in pDC by these TLR7 stimuli was completely suppressed by incubation with phosphorothioate ODN, consistent with the inhibitory effects seen in TLR7-transfected cells. There was no apparent sequence-dependence in the inhibition of TLR7-mediated signaling by phosphorothioate ODN in transfected cells or in pDC, in contrast to the sequence-selective stimulatory effect seen on TLR8. The combination of ODN and loxoribine not only led to a switch from pDC-derived IFN- α to monocyte-derived IL-12 and TNF- α but also to secretion of IFN- γ from NK cells. Human NK cells lack TLR7 and TLR8 expression (31), so that stimulation of NK cell-derived IFN- γ appears to be an indirect effect, probably mediated by IL-12 (32). Taken together, a complete alteration of the profile of loxoribine-mediated immune effects was observed upon simple addition of certain oligonucleotides. Furthermore, these data point to a somewhat sequence-dependent effect with phosphorothioate T-rich ODN being the most efficient ODN that can be combined with TLR7-specific ligands to activate TLR8.

Some reports indicate that myeloid dendritic cells and monocytes express both TLR7 and TLR8 at the mRNA level (27-29). No data are available showing actual TLR protein expression in these cells. No direct stimulation of monocytes by loxoribine was detected in these experiments. In contrast, only the combination of loxoribine and oligonucleotide induced monocyte-derived cytokine production. These results argue against a functional expression of TLR7 in monocytes and point to TLR8 as the receptor targeted by oligonucleotide and loxoribine. Furthermore, loxoribine alone did not induce significant amounts of IL-12, although production of IL-12 in human PBMC induced by R-848 has been reported (29), which is consistent with the ability of R-848 to stimulate both TLRs (16). A clear distinction as to which TLR is activated in cells that may express both TLR7 and TLR8

with compounds stimulating both receptors is very difficult. Ito et al (33) reported that mDC produce IL-12 but not IFN- α when stimulated with R-848. In contrast, pDC do not produce IL-12 but do produce IFN- α (25) upon stimulation with R-848 or other TLR ligands indicating a TLR8-mediated induction of IL-12 in mDCs and a TLR7-mediated induction of IFN- α in pDCs. Even co-incubation of R-848 or loxoribine with oligo(dT)₁₇ ODN did not result in production of IL-12 by human pDC suggesting that this signal is not sufficient to activate the adequate pathways in pDC.

It is tempting to postulate a model where certain TLRs may possess a (possibly allosteric) regulatory site to which oligonucleotides could bind and function as effector molecules. In the case of TLR7, phosphorothioate oligonucleotides appear to act as antagonists. Therefore, oligonucleotide binding to TLR7 may inhibit either proper binding of a TLR7 ligand to its binding pocket or correct downstream signaling, e.g., by aggravating correct conformational changes within the receptor. Binding of T-rich oligonucleotides to TLR8, on the other hand, may function as an (allosteric) activator enabling increased binding of R-848 or other small molecule ligands like loxoribine to the active TLR8 binding pocket or increased downstream signaling. Whether there may be two binding domains involved or binding of small molecule ligand and effector ODN may occur at the same site cannot currently be determined. Allosteric enhancers are known for receptors from several different families. For example, Knoflach et al. (34) reported the identification of two classes of small molecules that behave as allosteric enhancers for the metabotropic glutamate receptor, a member of the G protein-coupled receptor family. Detailed analysis of receptor structure localized the enhancer binding site within the transmembrane domain. Other studies show that small molecules can act as allosteric enhancers for the muscarinic acetylcholine receptor (35). Certain 2-Aminothiophenes act as allosteric enhancers of Adenosine receptor A1 (36,37) by stabilizing the ligand-receptor-G-protein ternary complex and increasing association of unoccupied receptor and G-protein (38). Interestingly, Gao et al. (39) found a series of imidazoquinoline derivates to act as allosteric enhancers of agonist binding at human A3 adenosine receptors. Recently, Rutz et al. (40) demonstrated direct blockade of CpG ODN binding to purified TLR9 protein by the small molecules chloroquine and quinacrine using SPR biosensor analysis. Their findings indicate direct binding of these molecules to the extracellular domain of TLR9 acting as antagonists of TLR9-mediated signaling. Therefore, the described oligonucleotides and small molecules may directly bind to TLR7 and 8. Binding studies using isolated proteins and the respective receptor ligands could give further

insight into the exact mechanism of action of the combination of small molecule TLR ligands and oligonucleotides on TLR7 or TLR8. These findings may have important implications for understanding the molecular signaling mechanisms of the TLRs, as well as for pharmaceutical research and drug development. It has been demonstrated, according to the invention, that the signaling activity of two members of the TLR family, TLR8 and TLR7, can be manipulated by the presence or absence of certain oligonucleotides. Taken together, these results suggest new ways to modify an immune response using loxoribine (or other small molecules) in combination with certain oligonucleotides. By combination of these molecules it is possible to alter the cytokine profile of the TLR small molecule ligand by redirecting its signaling activity to a different TLR. An altered or enforced immune response could be beneficial for the treatment of a variety of diseases.

TABLE 1

Table 1: Comparison of synergistic effects of different ODN on R-848 activity.

HEK293 cells expressing hTLR8 and a NF- κ B reporter construct were incubated with 50 μ M of R-848 in the absence or presence of 5 μ M of the indicated ODN. NF- κ B stimulation by R-848 in the absence of ODN was set to 100% and effect on R-848 activity was calculated accordingly. Values represent the mean (\pm SD) of 2-4 experiments. "N" represents A, C, G or T in random order, "*" depicts a phosphorothioate backbone, "—" depicts a phosphodiester backbone.

TABLE 2

SEQ ID NO:

T2	T*T
T4	T*T*T*T
T6	T*T*T*T*T*T
T8	T*T*T*T*T*T*T
T10	T*T*T*T*T*T*T*T
T12	T*T*T*T*T*T*T*T*T
T14	T*T*T*T*T*T*T*T*T*T
T17	T*T*T*T*T*T*T*T*T*T*T*T

1
1
1
10
19
20
3

SEQ ID NO:

C17	C*C*C*C*C*C*C*C*C*C*C*C
C7T2C8	C*C*C*C*C*C*T*T*C*C*C*C*C*C
C6T4C7	C*C*C*C*C*C*T*T*T*T*C*C*C*C*C
C5T6C6	C*C*C*C*C*T*T*T*T*T*C*C*C*C*C
C4T8C5	C*C*C*C*T*T*T*T*T*T*C*C*C*C*C
C3T10C4	C*C*C*T*T*T*T*T*T*T*T*C*C*C*C
T17	T*T*T*T*T*T*T*T*T*T*T*T*T*T

13
8
7
6
2
1
3

* indicates a phosphorothioate bond

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Equivalents

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as a single illustration of one aspect of the invention and other functionally equivalent embodiments are within the scope of the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The advantages of the invention are not necessarily encompassed by each embodiment of the invention.

All references, patents and patent publications that are recited in this application are incorporated by reference in their entirety herein.

What is claimed is:

Claims

1. A method for stimulating a TLR 8 mediated immune response comprising administering to a subject in need thereof a TLR 7/8 ligand and an adaptor oligonucleotide in an amount effective to stimulate a TLR 8 mediated immune response.
2. A method for redirecting a TLR7-mediated immune response to a TLR 8 mediated immune response comprising administering to a subject experiencing a TLR7-mediated immune response an adaptor oligonucleotide in an amount effective to redirect a TLR7-mediated immune response to a TLR8-mediated immune response.
3. The method of claim 1 or 2, wherein the TLR 7/8 ligand is a TLR7 specific ligand.
4. The method of claim 1 or 2, wherein the TLR7 specific ligand is a C8-substituted guanosine.
5. The method of claim 4, wherein the C8-substituted guanosine is 7-allyl-7,8-dihydro-8-oxo-guanosine (loxoridine), 7-thia-8-oxoguanosine (immunosine), 8-mercaptoguanosine, 8-bromoguanosine, 8-methylguanosine, 8-oxo-7,8-dihydroguanosine, C8-arylamino-2'-deoxyguanosine, C8-propynyl-guanosine, C8- and N7- substituted guanine ribonucleosides, 7-methyl-8-oxoguanosine, 8-aminoguanosine, 8-hydroxy-2'-deoxyguanosine, 7-deaza-8-substituted guanosine, and 8-hydroxyguanosine.
6. The method of claim 4, wherein the C8-substituted guanosine is loxoridine.
7. The method of claim 3, wherein the TLR7 specific ligand is 7-deaza-guanosine.
8. The method of claim 3, wherein the TLR7 specific ligand is 6-amino-9-benzyl-2-(3-hydroxy-propoxy)-9H-purin-8-ol, or 6-amino-9-benzyl-2-butoxy-9H-purin-8-ol.

9. The method of claim 1 or 2, wherein the TLR 7/8 ligand is a TLR 8 specific ligand.

10. The method of claim 1 or 2, wherein the TLR 7/8 ligand is a TLR 7 ligand and a TLR 8 ligand.

11. The method of claim 10, wherein the TLR 7/8 ligand is an imidazoquinoline.

12. The method of claim 11, wherein the imidazoquinoline is imidazoquinoline amine, imidazopyridine amine, 6,7-fused cycloalkylimidazopyridine amine, 1,2 bridged imidazoquinoline amine, R-848 (S-28463 or resiquimod), 4-amino-2ethoxymethyl- α , α -dimethyl-1H-imidazo[4,5-c]quinolines-1-ethanol, 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine (R-837 or Imiquimod), or S-27609.

13. The method of claim 11, wherein the imidazoquinoline is R848.

14. The method of claim 1 or 2, wherein the adaptor oligonucleotide comprises an unmethylated CpG motif.

15. The method of claim 1 or 2, wherein the adaptor oligonucleotide does not comprise an unmethylated CpG.

16. The method of claim 1 or 2, wherein the adaptor oligonucleotide comprises 5' N-TTTTT-N 3', where N is any nucleotide.

17. The method of claim 1 or 2, wherein the adaptor oligonucleotide comprises 5' N-TTTTTT-N 3', wherein N is any nucleotide.

18. The method of claim 1 or 2, wherein the adaptor oligonucleotide is a dT homopolymer.

19. The method of claim 1 or 2, wherein the adaptor oligonucleotide comprises a phosphorothioate backbone modification.

20. The method of claim 1 or 2, wherein the TLR 7/8 ligand and the adaptor oligonucleotide are administered separately.

21. The method of claim 1 or 2, wherein the TLR7/8 ligand and the adaptor oligonucleotide are conjugated to each other.

22. The method of claim 1 or 2, wherein the adaptor oligonucleotide is a DNA.

23. The method of claim 1 or 2, wherein the adaptor oligonucleotide is an RNA.

24. The method of claim 1 or 2, further comprising administering an antigen to the subject.

25. The method of claim 1 or 2, wherein the subject has an infection.

26. The method of claim 1 or 2, wherein the subject has cancer.

27. The method of claim 1 or 2, wherein the subject has allergy or asthma.

28. A composition comprising

a TLR 7 specific ligand selected from the group consisting of
6-amino-9 benzyl-2-(3-hydroxy-propoxy)-9H-purin-8-ol and
6-amino-9-benzyl-2-butoxy-9H-purin-8-ol, and
an adaptor oligonucleotide.

29. A composition comprising

a TLR 8 specific ligand and
an adaptor oligonucleotide.

30. The composition of claim 28 or 29, wherein the adaptor oligonucleotide comprises an unmethylated CpG motif.

31. The composition of claim 28 or 29, wherein the adaptor oligonucleotide does not comprise an unmethylated CpG.

32. The composition of claim 28 or 29, wherein the adaptor oligonucleotide comprises 5' N-TTTT-N 3', where N is any nucleotide.

33. The composition of claim 28 or 29, wherein the adaptor oligonucleotide comprises 5' N-TTTTTT-N 3', wherein N is any nucleotide.

34. The composition of claim 28 or 29, wherein the adaptor oligonucleotide is a dT homopolymer.

35. The composition of claim 28 or 29, wherein the adaptor oligonucleotide comprises at least one phosphorothioate internucleotide linkage.

36. The composition of claim 28 or 29, wherein the adaptor oligonucleotide is a DNA.

37. The composition of claim 28 or 29, wherein the adaptor oligonucleotide is an RNA.

38. The composition of claim 28 or 29, wherein the adaptor oligonucleotide and the TLR 7 specific ligand or the TLR 8 specific ligand are conjugated to each other.

39. The composition of claim 28 or 29, further comprising an antigen.

40. The composition of claim 28 or 29, wherein the composition is a pharmaceutical preparation.

41. A method for identifying a TLR8 ligand comprising
contacting a TLR8-expressing cell with a test ligand in the presence and absence of an
adaptor oligonucleotide, and
measuring stimulation of the TLR8-expressing cell in response to the test ligand in the
presence and absence of the adaptor oligonucleotide,
wherein a TLR8 ligand is identified by an increased stimulation in the presence of the
adaptor oligonucleotide.

42. A method for identifying an adaptor oligonucleotide comprising
contacting a TLR8-expressing cell with a TLR7 ligand in the presence and absence of
a test adaptor oligonucleotide, and
measuring stimulation of the TLR8-expressing cell in response to the TLR7 ligand in
the presence and absence of the test adaptor oligonucleotide,
wherein an adaptor oligonucleotide is identified by increased stimulation of the TLR8-
expressing cell in the presence of the adaptor oligonucleotide.

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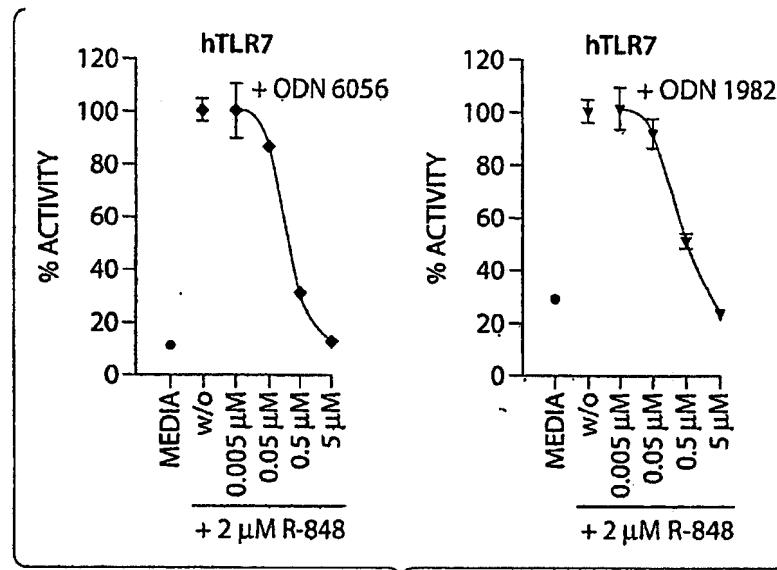


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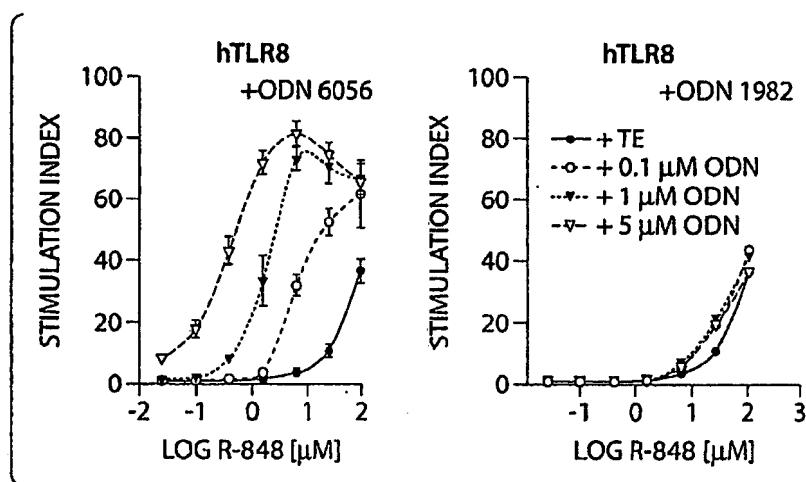


Fig. 1B

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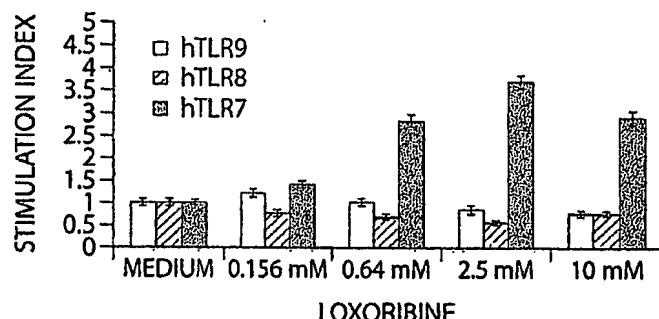


Fig. 2A

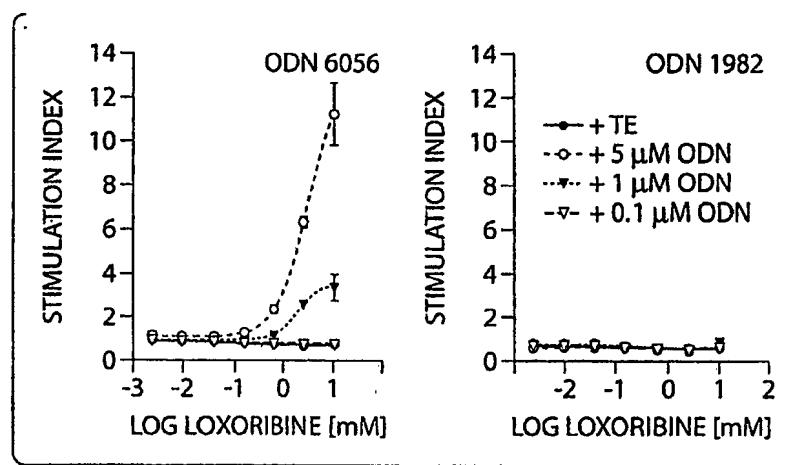


Fig. 2B

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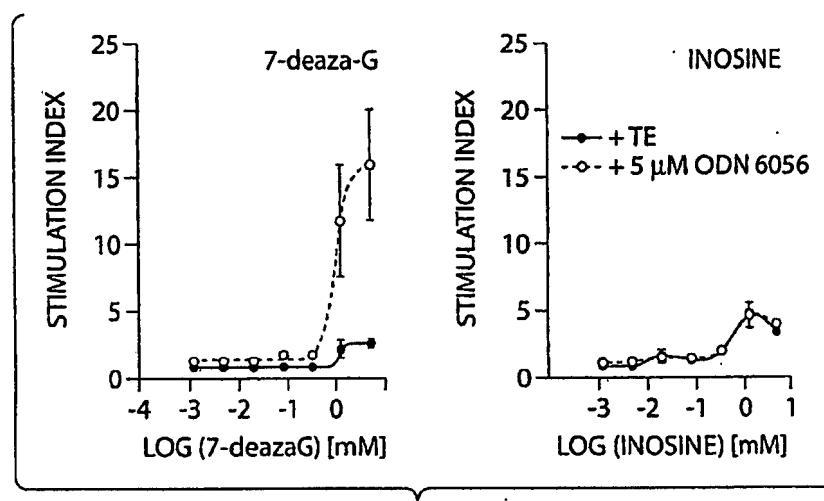


Fig. 2C

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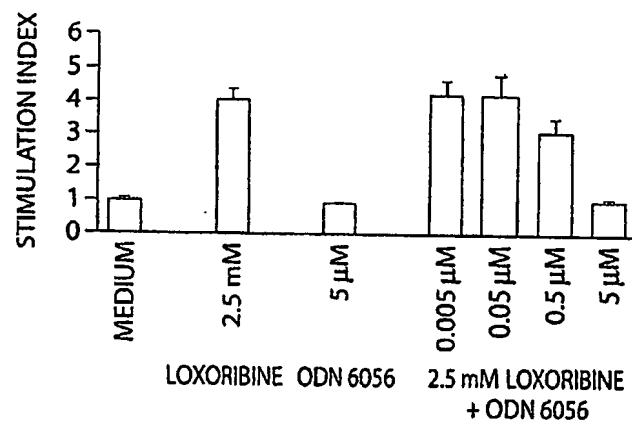


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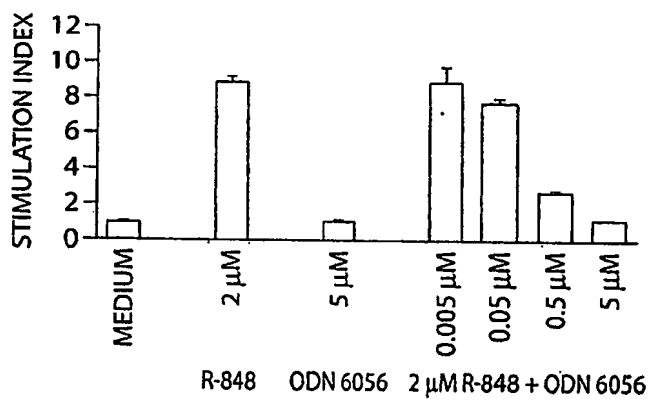


Fig. 3B

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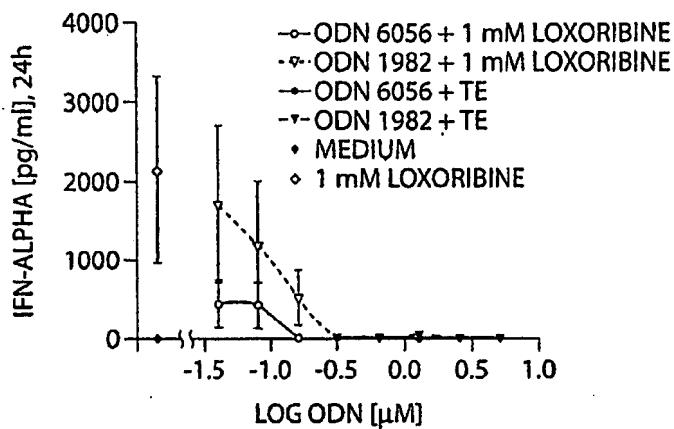


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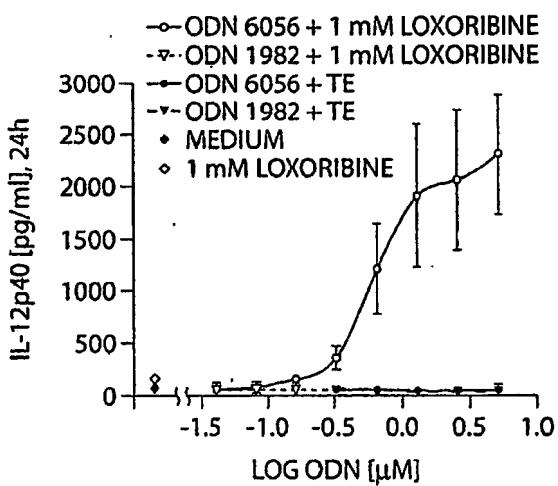


Fig. 4B

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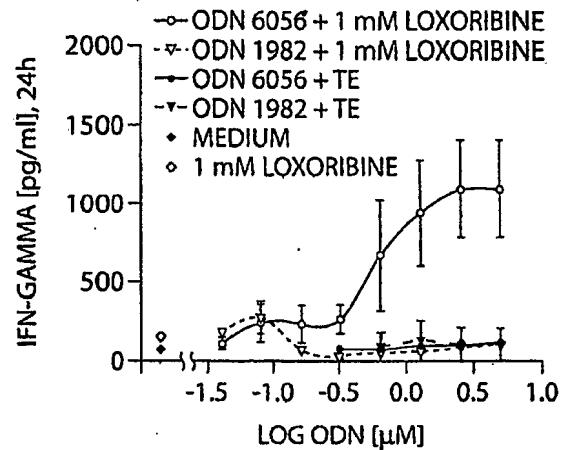


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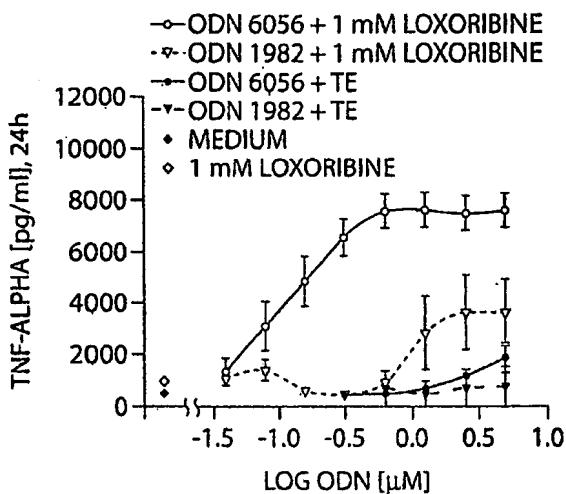


Fig. 4D

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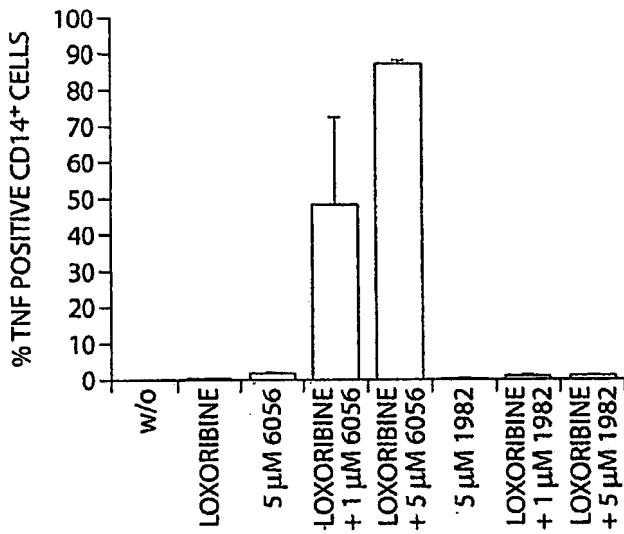


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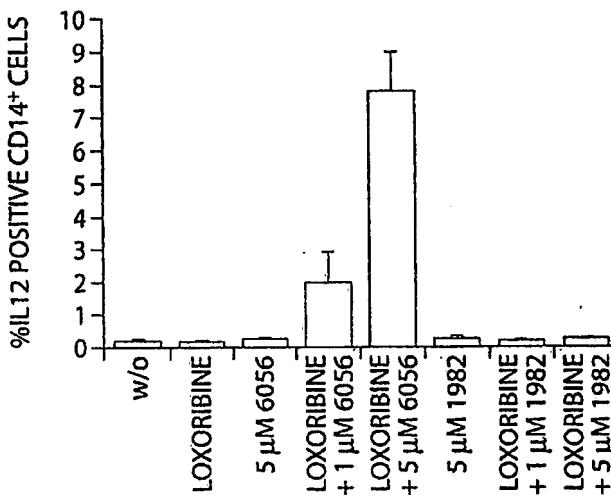


Fig. 5B

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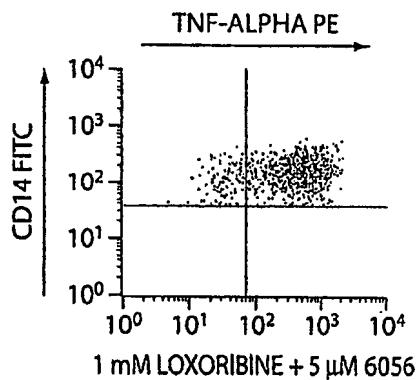


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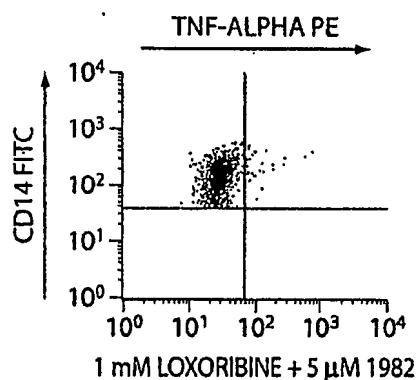


Fig. 5D

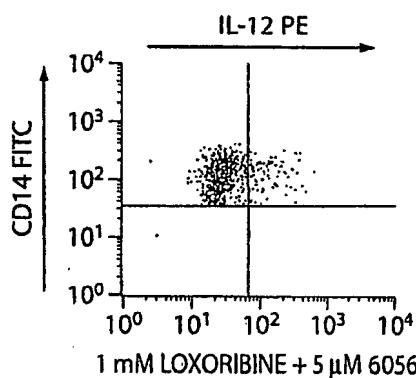


Fig. 5E

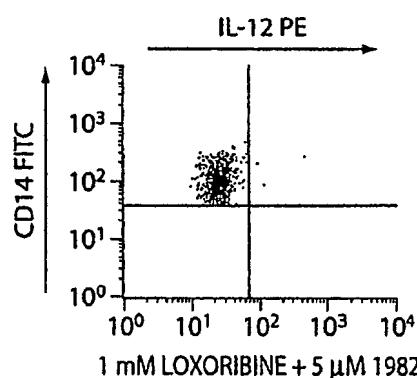


Fig. 5F

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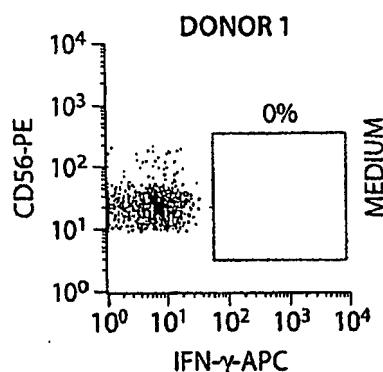


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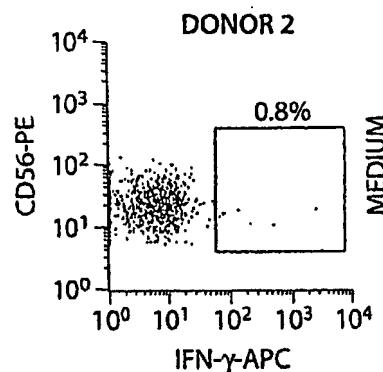


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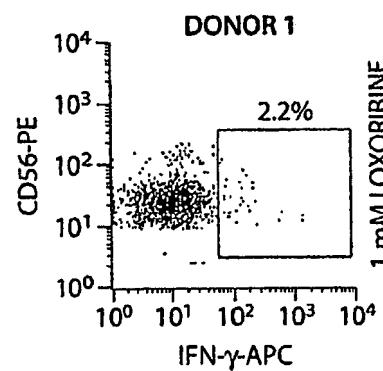


Fig. 6C

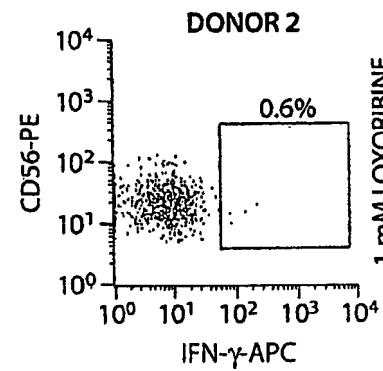


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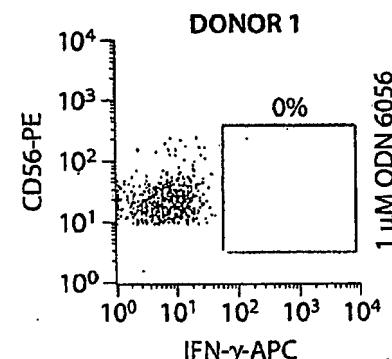


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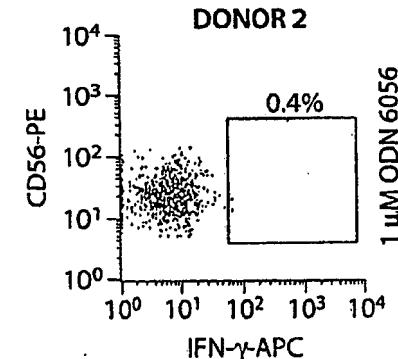
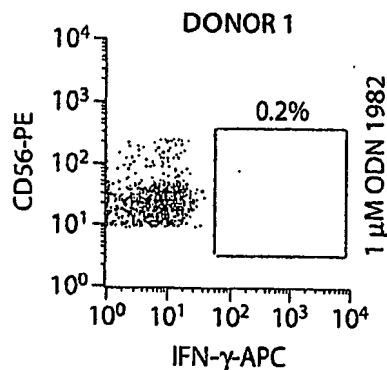
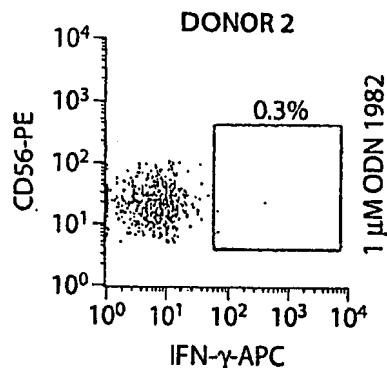
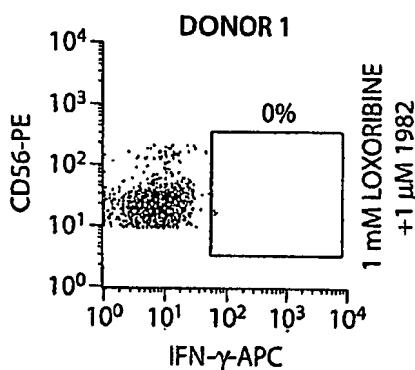
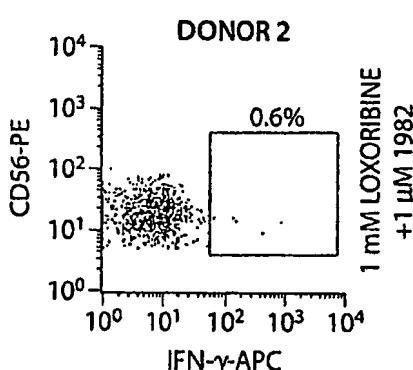
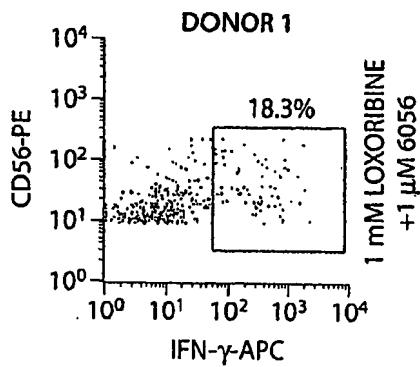
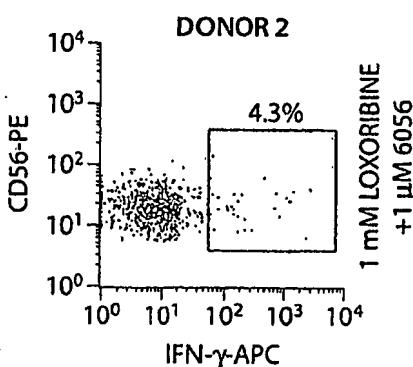


Fig. 6F

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**Fig. 6G****Fig. 6H****Fig. 6I****Fig. 6J****Fig. 6K****Fig. 6L**

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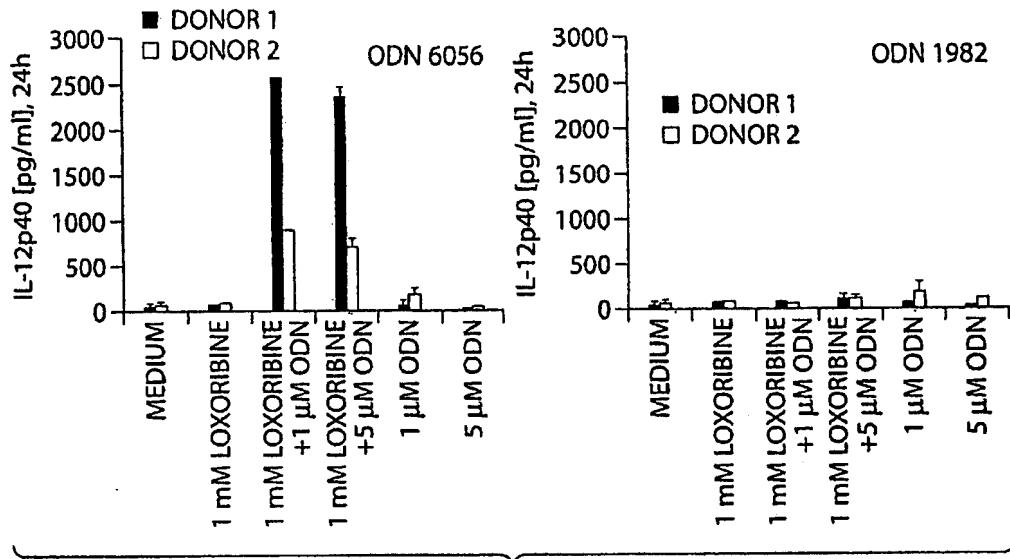


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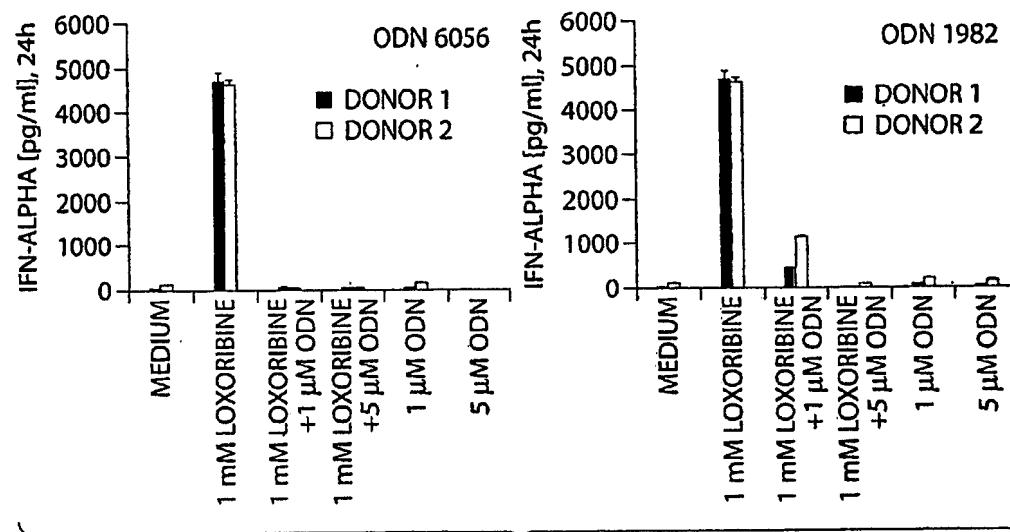


Fig. 7B

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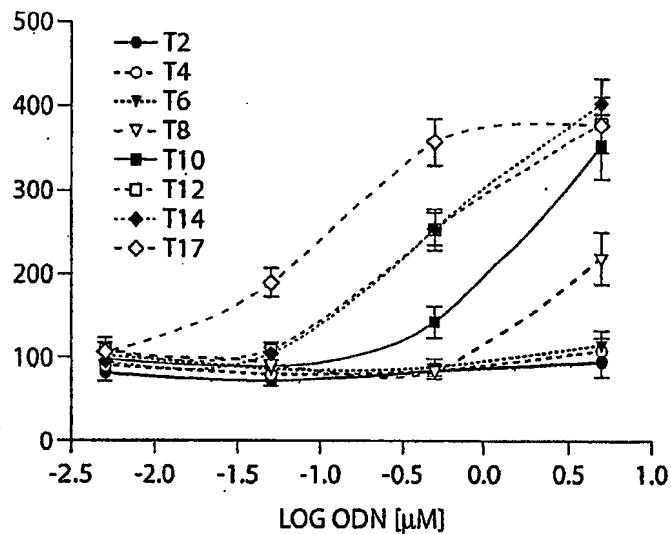


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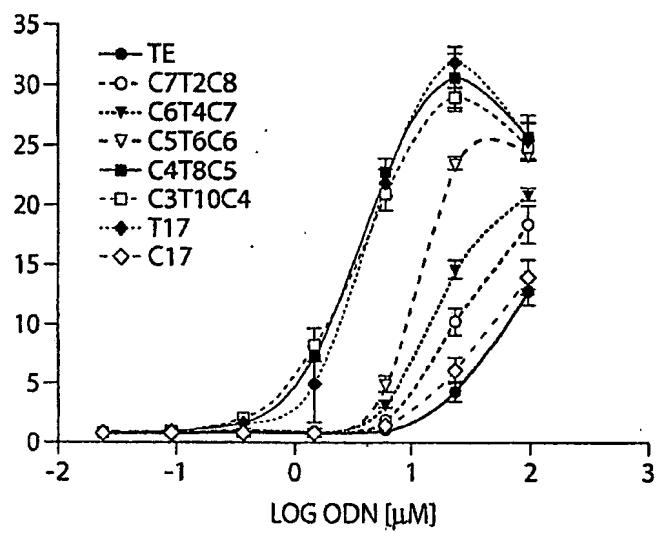


Fig. 8B

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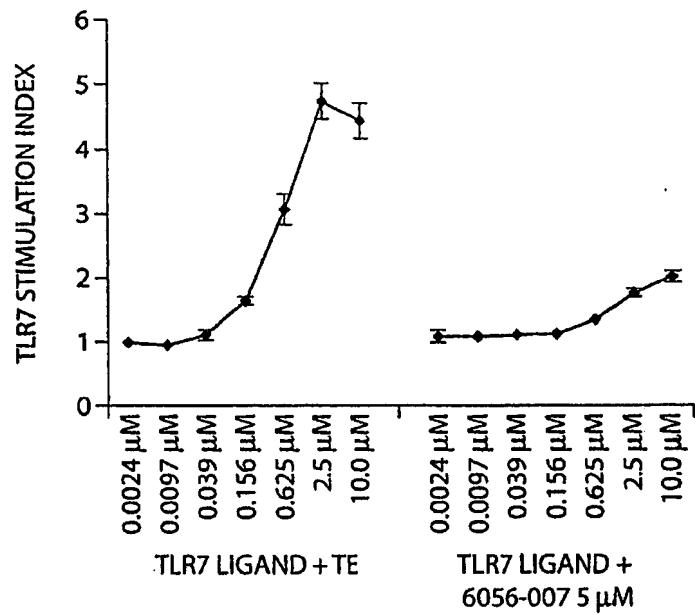


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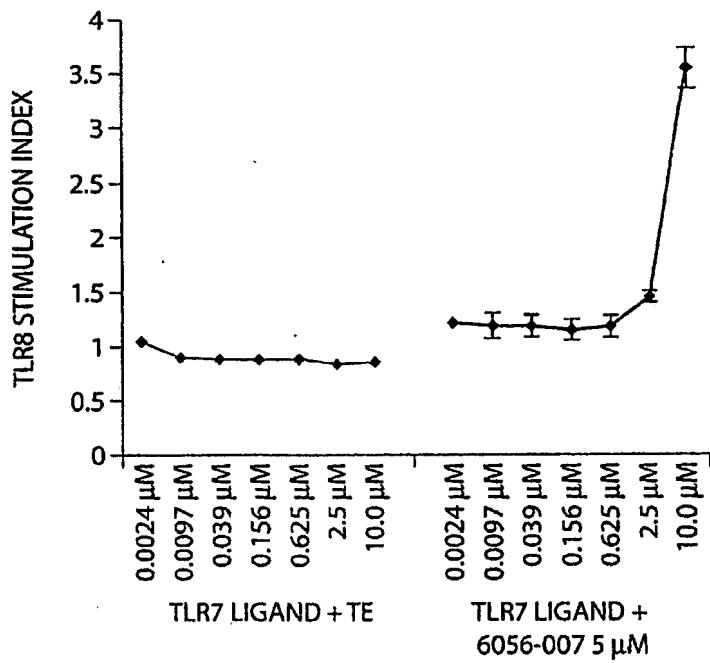


Fig. 9B

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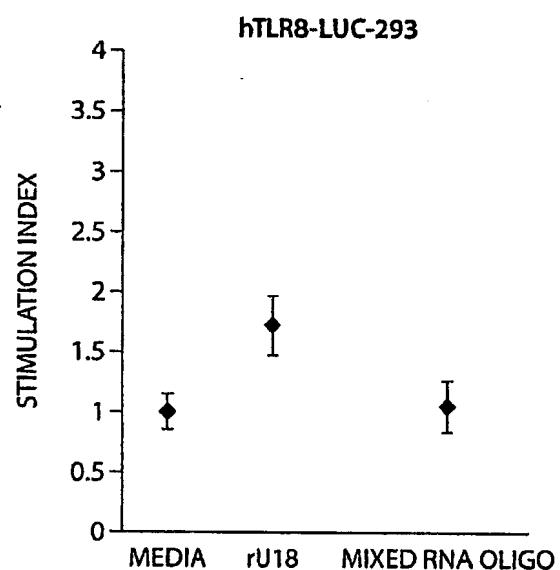


Fig. 10

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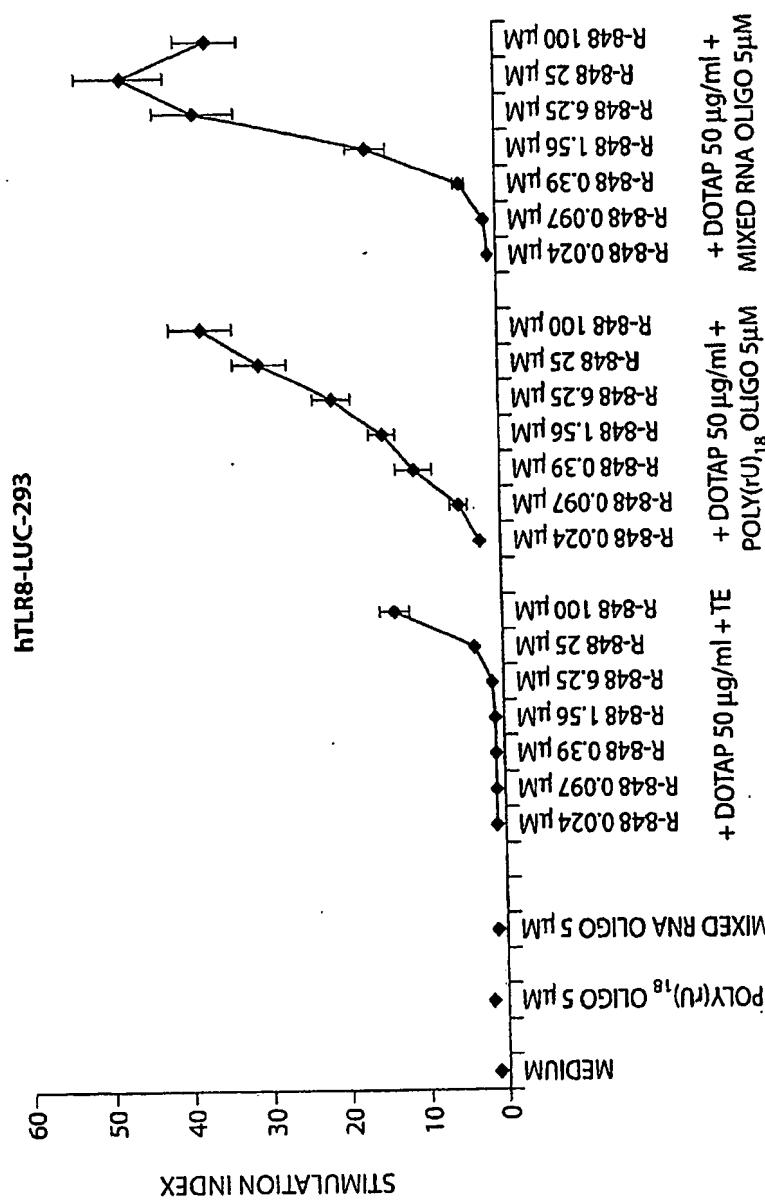


Fig. 11

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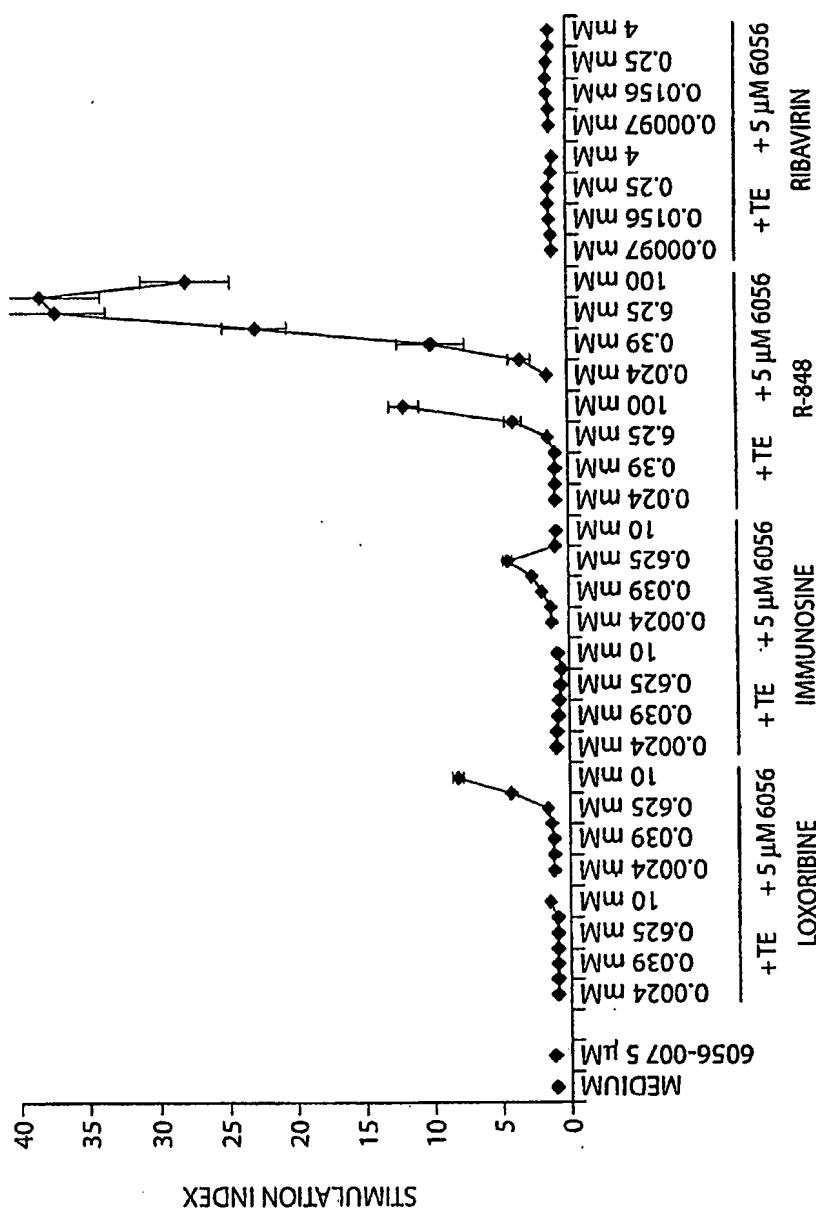
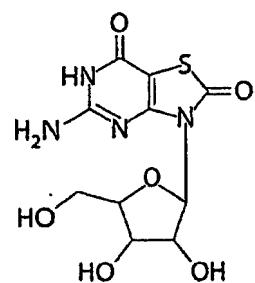


Fig. 12

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IMMUNOSINE

Fig. 13A

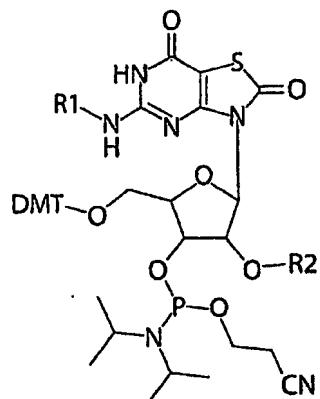


Fig. 13B

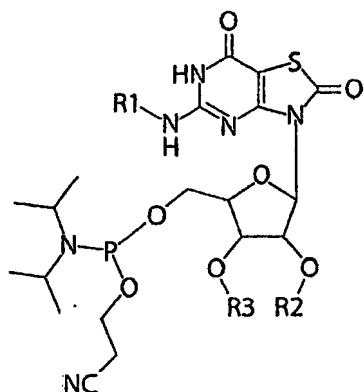


Fig. 13C

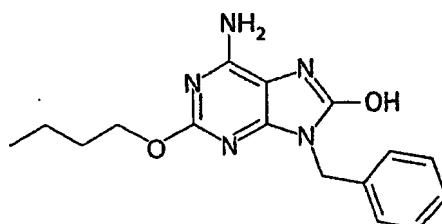


Fig. 13D

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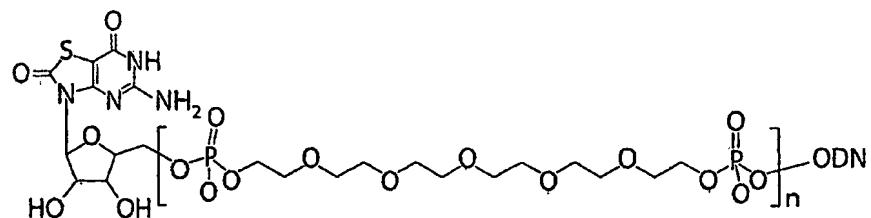
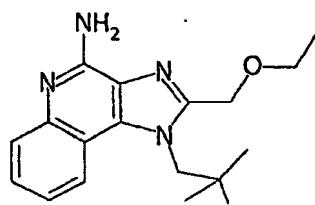


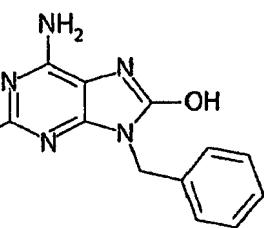
Fig. 14



R-848

ATTACHMENT POINT

ATTACHMENT POINT



CL-029

Fig. 15A

Fig. 15B

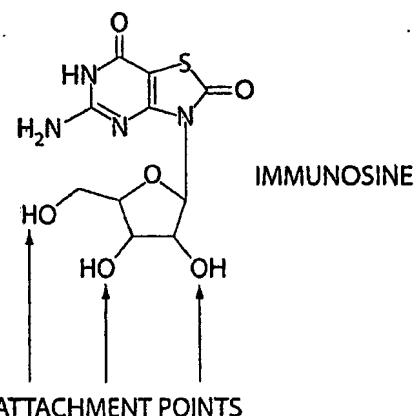


Fig. 15C

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Coley Pharmaceutical GmbH
Jurk, Marion
Vollmer, Jorg
Krieg, Arthur M
Uhlmann, Eugen
Noll, Bernhard O

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